

STUDIES ON ENZYME ACTION

XXXVIII. THE ESTER HYDROLYZING ACTIONS OF THE WHOLE EEL

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The systematic study of the ester hydrolyzing enzymes of various animal materials has been described in previous communications¹. Results obtained with the eel are presented in this paper

Experimental Methods

Adult eels whose weights ranged from 86 gm to 337 gm were used. Immediately after being killed, they were passed twice through a meat chopper. If the solid material was to be tested directly, 0.67 gm portions were weighed into flasks and 15 cc. water and 1 cc. toluene added to each. If extracts were to be tested, the requisite amounts of water or solution were added to the weighed solid, allowed to extract overnight at room temperature, filtered through paper, suitably diluted, and 15 cc portions used for the enzyme tests. In every enzyme test the concentration of the eel, in the form of original solid, or of the eel extract corresponded to 44.4 mg of original material per cc. of mixture or of solution tested. Because of the increases in volume in the dialysis experiments it was necessary to plan for suitable adjustment of the concentrations of the original extracts. Toluene was present throughout in every experiment.

Dialysis was carried out in collodion bags at 14-17° for 15 to 24 hours, either against tap water where the flow was comparatively rapid and the outside liquid not recovered, or against definite volumes

¹ Cf especially *J Biol Chem* 1924, lxx, 183, 213, 1924-25, lxxii, 687, 697; *J Gen Physiol*, 1925-26, viii, 75, 1925-26, ix, 651; *J Am. Chem Soc* 1924, xlii, 1885; *J Cancer Research*, 1925, ix, 105, 1926, x, 146.

of distilled water with constant stirring and where the outside liquid was used for enzyme tests

The enzyme tests were carried out as described in previous communications ten different esters were used, as shown in the figures and in the table, 3.4 milli-equivalents of each ester, incubation for 22 hours at 37-38°, titration with 0.1 N sodium hydroxide solution with phenolphthalein as indicator, and blanks and duplicates in every case

Results

The relative hydrolyzing actions of the solid and different extracts on the ten different esters are shown in Figs 1 and 2. The averages of three closely agreeing series of experiments are given in each curve, the actions on phenyl acetate being largest and placed equal to 100 in each case

The curves of the relative actions of the eel solids and extracts showed no striking differences either when tested directly or after dialysis against tap water. Minor differences, however, were indicated in the two figures

The absolute enzyme actions (actual titration values corrected for blanks), are not given in detail for these experiments. Only the general behavior will be stated. The solid material gave the largest actions, the undialyzed aqueous and 50 per cent glycerol extracts gave much the same actions but somewhat less than the solid, while the undialyzed 10 per cent sodium chloride extracts gave the smallest actions. On dialysis, the absolute actions of the various extracts decreased except for the 10 per cent sodium chloride extracts where the actions on phenyl acetate, glyceryl triacetate, and methyl butyrate were larger for the dialyzed than for the undialyzed extracts

Table I shows the results of a complete experiment (Experiment F20) in which the enzyme tests were carried out with the solid eel material and the various extracts directly, on these same mixtures after dialysis against distilled water on the dialysates, and on the dialyzed mixtures to which the dialysates were added. The concentration of each mixture tested was equivalent to 44.4 mg of original material per cc of solution tested. The actual titration values, cor-

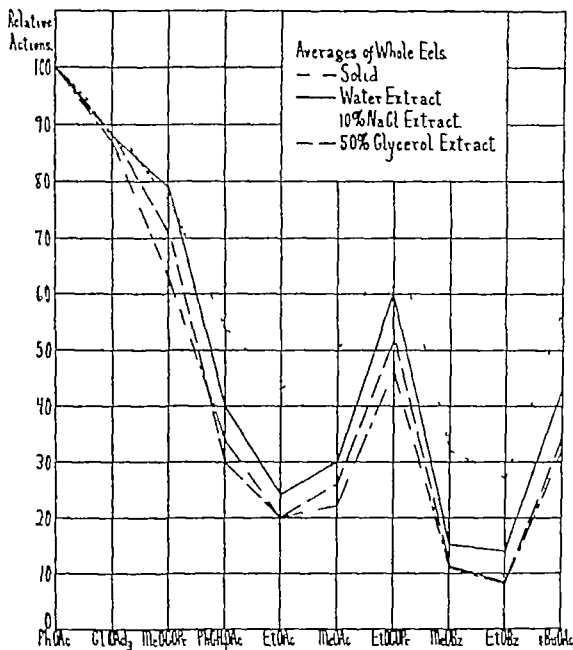


FIG. 1 Averages of relative ester hydrolyzing actions of solid eel preparation and of undialyzed extracts.

The 10 per cent NaCl extracts were diluted for the tests to contain 3.3 per cent NaCl the 50 per cent glycerol extracts to contain 16.7 per cent glycerol. The curves of the relative actions of the solids and of the glycerol extracts were essentially the same compared to these curves the aqueous extracts showed somewhat greater actions on the esters following phenyl acetate and glyceryl triacetate, and the 10 per cent NaCl extracts considerably greater for the last seven esters in comparison with the first three.

rected for blanks, are given. These represent the number of tenths of milli-equivalents of esters hydrolyzed by the enzyme material under the conditions of the tests.

The absolute values are of interest in connection with the curves of relative actions shown in *Fig. 1*. The figures in italics were calculated from the results of the separate experimental values given in the table.

One striking result is apparent. The calculated sums of the actions of dialysate and dialyzed solution in a number of cases are less than the actions found experimentally when the two were mixed. The dialysates by themselves showed practically no action except on phenyl acetate and glyceryl triacetate and even here the actions were small. The greater actions found experimentally as compared with the calculated include the following: (f) Dialyzed solid plus dialysate for the butyrates, benzyl acetate, and isobutyl acetate, (g) dialyzed solid plus liquid filtered from dialyzed solid plus dialysate for all the esters except phenyl acetate and glyceryl triacetate, but especially the butyrates, (h) liquid filtered from dialyzed solid plus dialysate, especially for the butyrates and isobutyl acetate, (t) dialyzed glycerol extract plus dialysate, small differences for a number of the esters.

These separations of certain of the enzyme materials into two parts, one of which is much less active than the original mixture and the other practically inactive, while the mixture is considerably more active than the sum of the two separate actions, are of interest. Such separations have been reported a number of times with various enzymes and the inactive portion termed "co-enzyme." The results presented here show that the solvent used influences the possibility of the separation and that the action of the co-enzyme may be very different for different substrates. No explanation is at hand for the action of the co-enzyme, but its greater influence on the hydrolysis of the butyric esters may possibly be of significance.

The absolute actions given in Table I supplement the relative actions shown in *Figs. 1 and 2*.

The ester hydrolyzing actions of the eel preparations were studied also at 14-17°. It was found that the actions on some of the esters in 22 hours were greater at the lower temperature than at 37-38°. This was shown to be due, in the main, to the combination, or opposing

b) 50 per cent glycerol extract.	3.96	3.25	2.62	1.16	0.76	0.92	1.92	0.41	0.31	1.14
c) Glycerol extract dial. w/ dist. water	3.04	2.54	2.13	1.05	0.59	0.73	1.63	0.34	0.28	1.08
d) Dialysate from glycerol extract.	0.57	0.25	0.11	0.03	0.01	0.04	0.06	0.04	0.01	0.00
e) Extract (p) + dialysate (r)	4.17	3.74	2.65	1.21	1.03	1.09	2.01	0.49	0.31	1.29
f) Extract (p) + dialysate (r) (calc.)	4.53	3.50	2.73	1.19	0.77	0.96	1.98	0.45	0.32	1.14
g) Dial. extract (q) + dialysate (r)	3.96	3.13	2.41	1.06	0.71	0.88	1.83	0.39	0.35	1.16
h) Dial. extract (q) + dialysate (r) (calc.)	3.61	2.79	2.24	1.05	0.60	0.77	1.69	0.38	0.29	1.08

natures, of two actions, one, the different rates of inactivation of the enzyme at the two temperatures, and the other, the different hydrolytic actions of the enzyme at the same two temperatures. These results, as well as others bearing on the problem of enzyme actions at different temperatures have been presented elsewhere,² and will therefore only be referred to in this connection.

SUMMARY

The hydrolyzing actions of various preparations of the adult eel were studied on ten esters in the usual way. The results are presented in the form of curves for the relative actions and in a table for the absolute actions obtained in one complete experiment.

The separation of the enzyme material in some cases into an active portion and a co-enzyme, the mixture showing greater actions on some esters than the sums of the individual actions, is described and discussed.

² Noyes, H. M., Lorberblatt, I., and Falk, K. G., *J. Biol. Chem.*, 1926, lxxviii, 135.

MICRURGICAL STUDIES IN CELL PHYSIOLOGY

II. THE ACTION OF THE CHLORIDES OF LEAD, MERCURY, COPPER, IRON, AND ALUMINUM ON THE PROTOPLASM OF AMOEBA PROTEUS

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The effects on protoplasm of some of the cations found in physiological systems have been reported in a previous communication (1). This study utilized the micrurgical technique and the advantages of such a method over that of simple immersion were pointed out in detail. Only by combining the results of injecting substances into the living cell with those obtained by the immersion method can one obtain proper conceptions of such important physiological problems as those of permeability, site of toxic action, antagonism of ions, and protoplasmic consistency.

Our knowledge of the action of heavy metals has been limited because of the difficulty in localizing the effect of salts in definite parts of the cell. The rapidity with which a cell or tissue reacts has usually been considered to mean ease of penetration (2, 3). But this is not necessarily the case. Even where some change has been noted inside the cell one cannot be certain that it might not be due to a surface effect which involved a secondary change within the interior or that it was not caused by the abstraction of some substance from the interior of the cell.

In a review of the work dealing with the action of disinfectants, especially HgCl_2 , Cohen (4) points out that previous contributions deal mainly with the course of the process and not with the mechanism concerned. The micrurgical method is especially adapted to a study of the mechanism of the reactions between salts and protoplasm because of the ease with which substances can be brought into direct contact with definite parts of the cells. The importance of many

of the metals in certain physiological (5-7) and pathological (8-10) problems suggested their study by means of micrurgy. With the aid of microdissection and injection, therefore, the effects of the chlorides of lead, mercury, copper, iron, and aluminum on *Amæba proteus* were investigated. It is the purpose of this paper to present the results of immersing and tearing amebæ in solutions of these salts and of injecting such solutions into these cells.

The manipulation of the apparatus, the general experimental technique, and the terms used, have been fully described in our former paper (1) and the reader is referred to that report for the details of the procedure. The only general modification of the method used in the experiments described in this paper was that in most of them fewer amebæ were used. This seemed justifiable because of the relatively constant quantitative results with several thousand amebæ from the same stock in former experiments.

I

Immersion Experiments

PbCl₂—PbCl₂ undergoes gradual hydrolysis in solution, with increase in acidity, and not until a dilution of M/5500 is reached can a solution be maintained at pH 5. If amebæ are immersed in decreasing concentrations of such solutions of PbCl₂, they undergo a slow change in shape. They gradually retract their pseudopodia and assume the elliptical form of the so called *Limax* type. The surface is stiffened and the ameba becomes very sluggish. Finally the cell becomes rounded and then dies. The curve labelled PbCl₂ in Fig 1 illustrates the relatively slow action of PbCl₂. It is toxic eventually in even very great dilutions. Amebæ cannot survive longer than 5 days until a dilution of M/22,000,000 is reached.

HgCl₂—Amebæ immersed in HgCl₂ die very rapidly compared to those in most of the other salts (Fig 1). In solutions stronger than M/8000 they are converted into small round masses. In solutions ranging from M/8000 to M/250,000 the plasmalemma breaks and the contents begin to scatter but solidify rapidly. Fig 1 shows that the curve of toxicity for HgCl₂ is relatively steep. A solution of M/1000 is toxic in 1 hour, but amebæ survive more than 5 days in M/250,000. That the immediate effect may not be due entirely to

the acidity which develops when HgCl_2 is dissolved in water is shown by the fact that amebæ can live normally in water at pH 6 but die rapidly in the presence of an $\text{M}/64,000$ solution of HgCl_2 of the same pH

CuCl_2 .—The reaction of an $\text{M}/1000$ solution of CuCl_2 is pH 5.5 1 day after the salt is dissolved. After 3 days the acidity increases to pH 4.8. Immersion of amebæ in these solutions of CuCl_2 causes the amebæ to become rounded and the contractile vacuole to increase in size. During the first 3 days of immersion amebæ die in a considerable range of dilutions (Fig 1). In solutions weaker than $\text{M}/4,100,000$ toxicity decreases abruptly

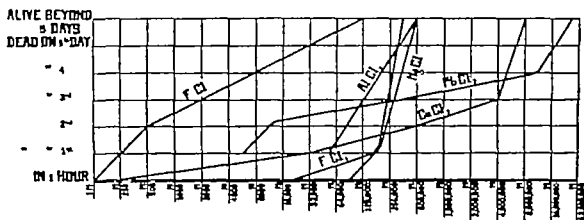


FIG 1 Viability of amebæ immersed in decreasing concentrations of salt solutions (PbCl_2 , HgCl_2 , CuCl_2 , FeCl_3 , FeCl_2 , AlCl_3)

FeCl_2 .—The acidity of solutions of FeCl_2 increases rapidly on standing, the greatest amount of change, however, occurring early. For example, a solution as dilute as $\text{M}/66,000$ changes from pH 6.2 to pH 5 in 4 days. These solutions of FeCl_2 cause immersed amebæ to become rounded and the crystalloid granules within the ameba become blackened. FeCl_2 is relatively non-toxic during the first 2 days of immersion after which time the toxicity increases rapidly (Fig 1). Thus, amebæ can live in a more dilute solution than $\text{M}/500$ for 2 days but cannot live for 5 days until an $\text{M}/128,000$ solution is reached.

FeCl_3 .—Solutions of FeCl_3 gradually increase in acidity (e.g. $\text{M}/325,000$ has a reaction of pH 6.2 when first made and is more acid

¹ In preparing solutions of FeCl_3 the molecular weight was taken as 540.44 ($\text{Fe}_2\text{Cl}_6 \cdot 12\text{H}_2\text{O}$)

than pH 5 in 5 days) FeCl_3 is much more toxic but produces the same visible effect as the salt of divalent iron. The amebæ become rounded and the crystalloid granules blacken. The maximum toxicity is approached after 1 day of immersion (Fig 1). Subsequently the curve of toxicity rises more steeply than for any of the other salts used in these experiments.

AlCl_3 —Solutions of AlCl_3 are acid in reaction over a considerable range (an $\text{M}/64,000$ solution has a pH of 5). This salt, in concentrations of $\text{M}/400$ and higher, causes the amebæ to become round rapidly. In solutions from $\text{M}/400$ to $\text{M}/32,000$ the amebæ continue to move about until their surfaces break. The most striking effect of the salt is the tremendous enlargement of the contractile vacuole (Fig 2). This occurs in concentrations of $\text{M}/2,000,000$ and stronger. The

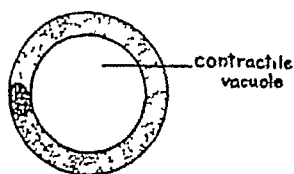


FIG 2 The effect of AlCl_3 on amebæ in injection ($\text{M}/32$ to $\text{M}/250$) and in immersion ($\text{M}/400$ to $\text{M}/2,000,000$) experiments

Note tremendous enlargement of contractile vacuole

granules are pushed against the plasmalemma by the distended vacuole. In very dilute solutions, in which the amebæ may live beyond 5 days, the large contractile vacuole may persist for 2 or 3 days but then decreases to its normal size and the ameba recovers completely. The curve of toxicity of AlCl_3 is fairly steep (Fig 1).

II

Injection Experiments

PbCl_2 —In solutions stronger than $\text{M}/1000$, PbCl_2 is changed rapidly into the insoluble carbonate by the abstraction of CO_2 from the air. The injection of PbCl_2 in concentrations ranging from $\text{M}/1000$ to $\text{M}/20,000$ (Fig 3) causes the gradual appearance of an irregular, glassy mass containing very few granules. The streaming movements in the rest of the ameba are very active and the glassy mass is

extruded. A second injection immediately after the first results in no solidification. If a large amount is introduced, the surface of the cell may break. If a second injection is made 20 minutes or more after the first, a second solidification and extrusion of the affected region may occur. Subsequent injections have no effect on the viability of the ameba, whether extrusions do or do not occur.

The effect of $PbCl_2$ in causing a delayed coagulation after the first injection and no coagulation after an immediate second injection, suggest that in the reaction between $PbCl_2$ and the protoplasm the lead uses up some cellular constituent which gradually forms anew in the cell. This harmonizes with the suggestion made by Aub and Rez-

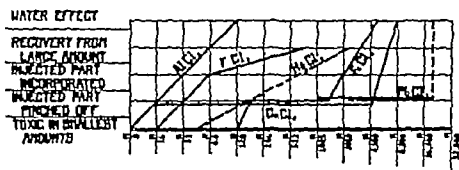


FIG 3 Recovery of protoplasm of amebæ from injection of decreasing concentrations of $PbCl_2$, $HgCl_2$, $CuCl_2$, $FeCl_3$, $FeCl_2$, and $AlCl_3$. The curve labelled $HgCl_2$ is represented by a broken line because no pinching off of the region injected occurs. The broken line in the curve labelled $PbCl_2$ indicates an abrupt transition from the pinching off of the coagulated region to the water effect. No intermediate stages are seen.

nikoff (11) that lead, in the small concentrations used in these experiments, unites with the phosphates or carbonates of the cell. The delay in the formation and the peculiar glassy appearance of the solidified mass in the cytoplasm also point to a different type of reaction between protoplasm and lead than those obtained with the other coagulating ions studied.

$HgCl_2$.—A solution of $1/5$ to $1/50$ $HgCl_2$ causes an immediate solidification of the internal protoplasm (Fig 3). With solutions of $1/100$ to $1/1600$ the surface breaks and some of the contents of the ameba flows out. Small amounts of $1/300$ $HgCl_2$ cause a disruption of the surface with subsequent recovery. Injections of small amounts of $1/600$ produce no break and the ameba readily recovers from the

breaks caused by larger injections. A moderate quantity of $M/1600$ causes no break but the ameba may pinch off the somewhat solidified injected region. An injection of an $M/2400$ solution even in large quantities causes no disruption of the plasmalemma.

The results indicate that both very high and very low concentrations of $HgCl_2$ act principally on the interior of the cell. Moderate concentrations erode the plasmalemma.

$CuCl_2$ —The injection of solutions of $CuCl_2$ causes a solidification of the affected region and a disintegration of the adjacent surface. With $M/16$ the ameba can pinch off the affected region (Fig. 3). Stronger solutions solidify the entire cell. Not until $M/8000$ is reached does this solidification process cease. Each successive injection of $CuCl_2$ in concentrations ranging from $M/16$ to $M/8000$ causes the pinching off of the solidified area with its disintegrated surface.

After the solidified area is pinched off, the remnant is apparently normal except for a temporary moderate enlargement of the contractile vacuole. Some enlargement of the contractile vacuole also occurs when very dilute solutions are injected with no resulting local solidification.

$FeCl_2$ —Solutions of $FeCl_2$ stronger than $M/32$ solidify the internal protoplasm with which they come into direct contact (Fig. 3). With $M/32$ the affected portion is usually pinched off. $M/64$ causes a quiescence and partial solidification of the injected region, which is subsequently reincorporated by the active portion of the ameba. Large amounts of $M/512$ may sometimes cause death, but moderate quantities of $M/32$ to $M/64$ as a rule are not fatal. The method by which the ameba constricts off the solidified part when $FeCl_2$ is injected is somewhat different from that observed with most other solidifying agents. Instead of a sharp constriction between the injured and healthy portions (1), the ameba forms a line of demarcation between the healthy and affected area and the living part flows around the solidified region so that the latter lies in a deep depression and is slowly extruded as the depression is everted (Fig. 4). The surface of the extruded region has no definite pellicle in some places. The living portion appears normal except for a slightly enlarged contractile vacuole.

$FeCl_3$ — $FeCl_3$ is much more toxic than the divalent chloride of iron to the internal protoplasm (Fig 3). A small injection of $M/160$ solidifies almost the entire ameba and the streaming movements of the unaffected region, which are apparently attempts to pinch itself off, soon cease and the whole cell is killed. From $M/320$ to $M/1280$ pinching off usually occurs after each injection. Occasionally, after the introduction of $M/320$ the ameba may incorporate the affected mass. With $M/5120$ an extrusion of a small mass from the injected region may occasionally occur after repeated injections. $FeCl_3$ causes the ameba to become sluggish and no typical water effect (1) is obtained until a dilution of $M/10,240$ is reached. Im

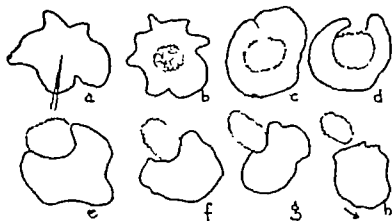


FIG 4. Injection of $M/32$ $FeCl_3$ into ameba, a before injection b solidification of the region injected c d e f and g extrusion by sliding around solidified area h complete separation of living remnant from dead area.

mediately after injection the vacuole of the ameba enlarges to some extent

$AlCl_3$.—The introduction of $AlCl_3$ has a very striking effect on the ameba. In strengths of $M/16$ and stronger the injected area is solidified (Fig 3). $M/8$ affects the entire ameba but when a solution of $M/16$ is introduced only the portion injected is solidified and is subsequently pinched off by the unaffected part of the ameba. The injection of $M/32$ also causes solidification, and pinching off may occur if the injection is made near the edge of the cell. However, in many such cases the ameba may reincorporate the affected mass after it is almost separated from the living portion, as though it were a foreign body. The solidified portion, held by a narrow band is finally en

gulfed by the ameba and is soon completely absorbed. The various steps of this process are illustrated in Fig 5. One of the most marked features of the injection of AlCl_3 in concentrations ranging from $M/32$ through $M/250$ is the tremendous enlargement of the contractile vacuole (Fig 2). This is identical with the results obtained in the immersion experiments. The ameba recovers from this condition, which may last from a few hours to several days, depending upon the concentration of AlCl_3 injected. The similarity in appearance of the internal protoplasm in immersion and injection experiments indicates a high degree of permeability which is in accord with the findings of Michaelis (12).

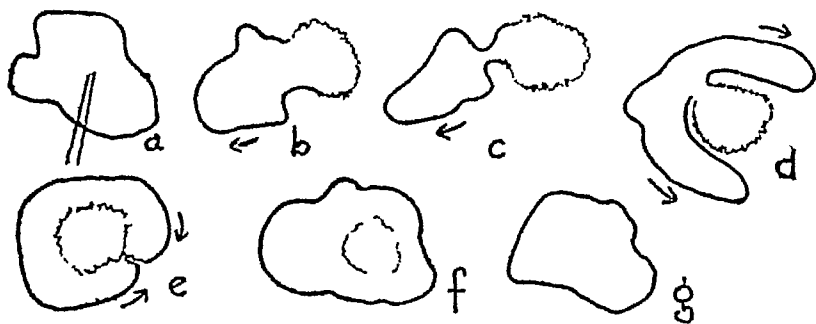


FIG 5 Injection of $M/32 \text{ AlCl}_3$ into ameba, *a*, before injection, *b* and *c*, uninjured portion flowing away from injected area and beginning to pinch it off, *d*, almost complete pinching off and beginning of engulfment of injured region, *e*, completion of engulfment, *f*, incorporation, *g*, return to normal state

III

Tearing Experiments

Because of the marked toxicity of even dilute solutions of the heavy metal salts to immersed amebæ, it is very difficult to maintain them alive and in good condition long enough to react visibly to a tearing operation. Thus, in HgCl_2 and FeCl_3 the rapidity of action of the salts is so great compared to that in salts like AlCl_3 and PbCl_2 that the amebæ are dead before the needles can be brought into use. Therefore the results of these experiments merely show the dilution at which the immersed amebæ remain alive long enough to react to a marked trauma of the needle. This, of course, varies with the rate of the action of the salt on the plasmalemma.

With this limitation in view the following table indicates the reparability of the torn surfaces of amebæ immersed in these salts

Salt.	Greatest concentration of salts in which repair of torn plasmalemma takes place.
AlCl_3	M/320
PbCl_2	M/1 000
FeCl_3	M/2 000
CuCl_2	M/3 200
FeCl_2	M/10 200
HgCl_2	M/24 000

DISCUSSION

These experiments present some evidence as to the actual mechanism involved in the effect on protoplasm of the salts tested. An attempt to group the entire action of all the heavy metals into a common process cannot be justified. There is, however, one common feature which merits consideration. All salts used in this work, PbCl_2 , HgCl_2 , CuCl_2 , FeCl_3 , FeCl_2 , and AlCl_3 , hydrolyze to form strong acids. Moreover, this process of hydrolysis takes place over an extended period of time. This has been studied for lead salts by von Ende (13). Coincident with the increase in acidity the amebæ gradually die and it is probable that this liberation of acid is at least one important factor in their death.

The marked toxicity of these salts when amebæ are immersed in them as compared with the results obtained when the salts are injected into the amebæ indicates that the lethal action of these substances is on the surface of the cell. In no case was the solution sufficiently acid to produce an effect on the internal protoplasm by its acidity alone. This has been shown by experiments previously reported (1), in which repeated injections of a solution of HCl at a reaction of pH 3 had no effect on the internal protoplasm but immersion in HCl at a reaction of pH 5 was lethal in a short time. This lends further support to the view that the action of the salt when injected is due to the cation alone, and that when the ameba is immersed in the solution the effect on the surface is due to the presence of the acid, which is being constantly produced, as

well as to the metal cation. It might, however, be suggested that the buffers in a cell can effectively neutralize any acid formed by hydrolysis. But the possibility must be considered that local effects may occur, for example on the surface, and cause irreversible changes before buffering of the acid takes place. This has been suggested by Aub and Reznikoff (11) as a possible mechanism in the action of lead on cells.

That the relative non-toxicity of the salts on the internal protoplasm may be due to their outward diffusion is not probable. No direct evidence for such outward diffusion was found in the case of NaCl (1), and the salts used in these experiments form much more stable compounds with protoplasm than does NaCl.

Some of these salts seem to have an effect on a specific part of the cell, depending upon their concentration. This is particularly true for HgCl_2 as has been found also by Bechhold (14) with red blood cells and by MacInnes (15) with *Aspergillus niger*. There is an indication, therefore, that various chemical combinations may be formed between the toxic substance and the different constituents of the cell depending upon the relative concentration of the toxic agent used. Krahé (16) suggests that the action of HgCl_2 is due not only to its ionization but, in certain concentrations, to its lipid solubility.

The gradual increase in toxicity of FeCl_2 in the immersion experiments may be associated with its gradual oxidation to the trivalent iron salt which is rapidly toxic. In this connection it is interesting to note that Buschke, Jacobssohn, and Klopstock (17) believe that the "oligodynamic" action of metals depends to a great extent on the ionization of their salts and on an oxidation process.

PbCl_2 , in the concentrations used in these experiments, probably acts by uniting with the phosphates or carbonates of the cell and thus liberating free acid. Such a secondary reaction is indicated by the slow rate of toxicity.

A striking feature brought out by these experiments is the greater variation in the viability of different amebæ in these solutions when compared to that which occurs in salts such as NaCl, KCl, CaCl_2 , and MgCl_2 (1).

In attempting to determine the mode of action of a toxic substance on a cell it is necessary to consider all the possible mechanisms in-

volved. A toxic agent may (a) affect the plasma membrane only, (b) affect both the plasma membrane and the internal protoplasm, (c) leave the plasma membrane unharmed and injure the internal protoplasm, or (d) may not enter the cell but affect it by abstracting a necessary constituent. In considering the third possibility (c), there is no evidence available so far, to support the belief that a substance may pass through the plasmalemma in a non toxic form and by some chemical alteration may change into a toxic form inside the cell or may be harmful to the internal protoplasm only. A consideration of the other possibilities, (a), (b), (d), suggests that a substance either abstracts a necessary constituent from the cell or primarily affects its surface. All visible evidence obtained so far points to the fact that toxic agents affect the surface of the immersed cell. So consistent is this result that the suggestion may be made that the maintenance of the surface membrane in a normal state is necessary for the life of the cell.

CONCLUSIONS

I *Plasmalemma*

1 The order of toxicity of the salts used in these experiments on the surface membrane of a cell, taking as a criterion viability of amebæ immersed in solutions for 1 day, is $\text{HgCl}_2 > \text{FeCl}_3 > \text{AlCl}_3 > \text{CuCl}_2 > \text{PbCl}_2 > \text{FeCl}_2$.

Using viability for 5 days as a criterion, the order of toxicity is $\text{PbCl}_2 > \text{CuCl}_2 > \text{HgCl}_2 > \text{AlCl}_3 > \text{FeCl}_3 > \text{FeCl}_2$.

2 The rate of toxicity is in the order $\text{FeCl}_3 > \text{HgCl}_2 > \text{AlCl}_3 > \text{FeCl}_2 > \text{CuCl}_2 > \text{PbCl}_2$.

3 The ability of amebæ to recover from a marked tear of the plasmalemma in the solutions of the salts occurred in the following order $\text{AlCl}_3 > \text{PbCl}_2 > \text{FeCl}_3 > \text{CuCl}_2 > \text{FeCl}_2 > \text{HgCl}_2$.

II *Internal Protoplasm*

4 The relative toxicity of the salts on the internal protoplasm, judged by the recovery of the amebæ from large injections and the range over which these salts can cause coagulation of the internal protoplasm is in the following order $\text{PbCl}_2 > \text{CuCl}_2 > \text{FeCl}_3 > \text{HgCl}_2 > \text{FeCl}_2 > \text{AlCl}_3$.

5 AlCl_3 in concentrations between $\text{M}/32$ and $\text{M}/250$ causes a marked temporary enlargement of the contractile vacuole FeCl_2 , FeCl_3 , and CuCl_2 produce a slight enlargement of the vacuole

6 PbCl_2 , in concentrations used in these experiments, appears to form a different type of combination with the internal protoplasm than do the other salts

III Permeability

7 Using the similarity in appearance of the internal protoplasm after injection and after immersion to indicate that the surface is permeable to a substance in which the ameba is immersed, it is concluded that AlCl_3 can easily penetrate the intact plasmalemma CuCl_2 also seems to have some penetrating power None of the other salts studied give visible internal evidence of penetrability into the ameba

IV Toxicity

8 The toxic action of the chlorides of the heavy metals used in these experiments, and of aluminum, is exerted principally upon the surface of the cell and is due not only to the action of the metal cation but also to acid which is produced by hydrolysis

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A SIMPLE ELECTRO-ULTRAFILTER.

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(Accepted for publication July 18 1926)

It is frequently desirable to free colloidal solutions of electrolytes but the process of ordinary dialysis is very slow and often cumbersome. Although the rate of diffusion of electrolytes out of the solution may be increased by means of electrophoresis, the limited choice of membranes available for use with the existing apparatus often leads to unequal rates of passage of the ions to the respective electrodes, and to greater or less marked changes in the hydrogen ion concentration of the solution subjected to dialysis.

We have encountered these difficulties in attempting to dialyze the solutions containing bacteriophage and have been successful in overcoming them by combining the principle of ultrafiltration with electrophoresis.

Our apparatus consists essentially of three concentric chambers, of which the middle one is formed by the collodion membranes deposited on the surfaces of two alundum thimbles (8 and 9, Fig 1, *a*). The other two chambers serve for removing the dialyzed electrolytes from the membrane by means of a stream of cold water.

The different parts of the dialyzing apparatus are as follows:

- (1) A glass tube¹ (3 mm. diameter) supplying distilled water from the reservoir (20) to the inner surface of the alundum thimble (8) carrying the positively charged membrane.
- (2) A rubber stopper securing the position of the glass tube (1) in place.
- (3) A glass T tube draining by suction into receptacle (21).
- (4) A rubber collar securing an air tight connection between the T tube (3) and the carbon (5).
- (5) A soft core arc lamp carbon 12 mm. in diameter and 150 mm. long bored

¹ This tube is slightly flared out at the lower end and carries a rubber washer to make an air tight connection at the lower end of the carbon so that at this point the glass tube is patent, but the hollow carbon (5) is not.

out to admit freely the glass tube (1) and connected with the positive wire of the electric current 60 mm from the slightly tapered lower end of the hollow carbon, a 2 mm hole permits the return flow of water from the inner chamber around the glass tube (1) and up to the collecting flask (21)

(6) A rubber stopper, No 13, shelved at its lower end so that a projection 10 mm deep and approximately 34 mm in diameter is left to accommodate the outer alundum thimble (9)

(7) A piece of rubber tubing making an air-tight connection between the carbon and the alundum thimble (8)

(8) A Norton alundum thimble, No 10472 RA 360, 17 mm in diameter and 80 mm long After this part of the apparatus has been assembled, a collodion membrane of the desired degree of permeability is deposited, under pressure, on the outer surface of the thimble (8) For this purpose the T-tube (3) is connected with the vacuum pump, the rubber tube at (1) is clamped off, and the thimble, while under negative pressure, is dipped into the solution of collodion in glacial acetic acid² for 60 seconds The connection with the vacuum pump is now broken, the excess of collodion is allowed to run off, and the coated surface is placed into running warm water to coagulate the collodion and to remove the acid The removal of the last traces of acid is most quickly accomplished by assembling the entire apparatus and using electrophoresis The membranes can be used repeatedly, provided they are kept in water when not in use Their permeability remains practically constant if they are not allowed to dry To renew the membrane it is necessary to remove the thimble, allow it to dry in air, and when thoroughly dry to incinerate it in the open flame

(9) Norton alundum thimble, No 6406 RA 360, 34 mm in diameter, 100 mm long The inner surface of the thimble must be glazed for a distance of 10 mm from the top, and the collodion membrane that is deposited upon its inner surface ends at this glazed rim For this purpose, the thimble is fitted by means of a suitably cut rubber stopper into a cylindrical funnel, and attached to the vacuum pump so as to apply negative pressure to the outside of the alundum The thimble is then filled with collodion for 60 seconds, the collodion is poured off, and warm water used as before The glazed surface will be found to slip easily over the projecting portion of the rubber stopper (6)

(10) Brass wire gauze, 120 mesh, tightly wrapped around the thimble (9) and held in place by clips connecting it with the lead (12) from the negative pole of the electric circuit

(11) A glass tube with rubber stopper at each end, carrying the negative lead (12)

(12) Lead from the negative pole of the circuit

(13) Glass tube carrying distilled water from the reservoir (19) to the middle chamber containing material for dialysis

² Bechhold, H, and Gutlohn, L, *Z ang Chem*, 1924, xxxvii, 494

(14) Thermometer

(15) A glass receptacle serving as an outer chamber and connected with receptacle (21) by a rubber tube forming a movable joint

(16) Overflow carrying away the ions migrating to the negative electrode.

(17) Stop-cock for emptying glass receptacle (15)

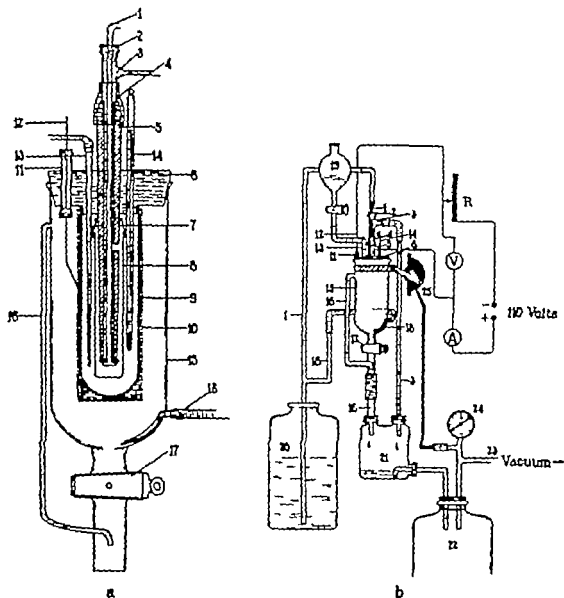


FIG 1

(18) Inlet for distilled water

(19) Fig 1 b Distilled water reservoir for middle chamber

(20) Distilled water reservoir for inner and outer chambers.

(21) Intermediary receptacle for dialyzates.

(22) Vacuum drainage carboy

(23) To vacuum pump

- (24) Vacuum gauge
- (25) Vacuum motor (windshield wiper)
- (A) Weston ammeter (Model 280)
- (V) Weston voltmeter, with switch (Model 280)
- (R) Sliding resistance, 650 ohms

The parts, as well as the assembled apparatus, may be obtained from Eimer and Amend, New York City

The high efficiency of this apparatus is due to several circumstances. The dialyzing surfaces are very large, considering the total capacity of the apparatus, the relative sizes of the membranes can be varied by using thimbles of appropriate sizes, the permeability of the membranes is easily varied by changing the density of the collodion used in coating the thimbles, the rate of dialysis is speeded up by a constant removal of the dialysate by a constant flow of water at each electrode, the constant flow of cold, distilled water at the electrodes permits the use of high voltage (110-115) without an excessive rise in temperature, the material subjected to dialysis may be kept from becoming concentrated by diluting it during dialysis, the whole dialyzing chamber is agitated, thus preventing the deposit of solids on the membrane. If necessary, the charges on the electrodes may be reversed by using a nickel screen instead of copper, and thus further adjustment in the relative rate of migration of ions may be accomplished.

In the sketch the outflow from both electrodes is mixed in the receptacles (21) and (22). If desirable, it is possible to collect the dialysates separately by leading tube (3) to a separate receptacle analogous to (21) and similarly connected to the vacuum pump.

The value of combining electrophoresis with ultrafiltration may be seen from the following. When 20 cc of broth were placed in the dialyzing chamber and the full current was turned on, the ammeter read 4.5 amperes. If this amount of broth were dialyzed without current, it would require 3 hours to increase its resistance to the passage of current sufficiently to give an ammeter reading of 0.016. When dialysis was combined with electrophoresis, a reading of 0.013 amperes was reached in 45 minutes.

INTERPRETATION OF THE LACTATION CURVE

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INTRODUCTION

The term lactation curve is used to refer to the curve representing the rate of milk secretion with advance in lactation. It has been known for some years that the group lactation curve for the dairy cow is of a descending exponential type. Sturtevant¹ first published herd data from which he concluded that the milk yield for any month showed a decrease of about 9 per cent as compared with the month preceding. Brody, Ragsdale, and Turner,² seeking to correlate the course of milk secretion with the laws governing the rate of chemical reactions, have expressed the lactation curve in the form of an exponential equation, $M_t = M_0 e^{-kt}$, in which M_t is the rate of yield at any time, t , and M_0 is the initial rate of yield. This is the type of equation representing the course of a monomolecular reaction in which M_0 would represent the initial amount of the substance undergoing such reaction and M_t would represent the amount of the substance remaining unchanged at any time, t . This similarity in form of the equations Brody *et al* interpret in favor of a limiting substance governing the maximum rate of milk secretion at any stage of lactation, the limiting substance undergoing monomolecular change which inactivates it so far as its effect on the rate of milk secretion is concerned.

The above results are based on the raw data of milk yield. It has been shown by Gaines and Davidson³ that the equation applies even more closely to the data of energy yield than it does to milk yield. While the equation is admirably adapted to describe the lactation

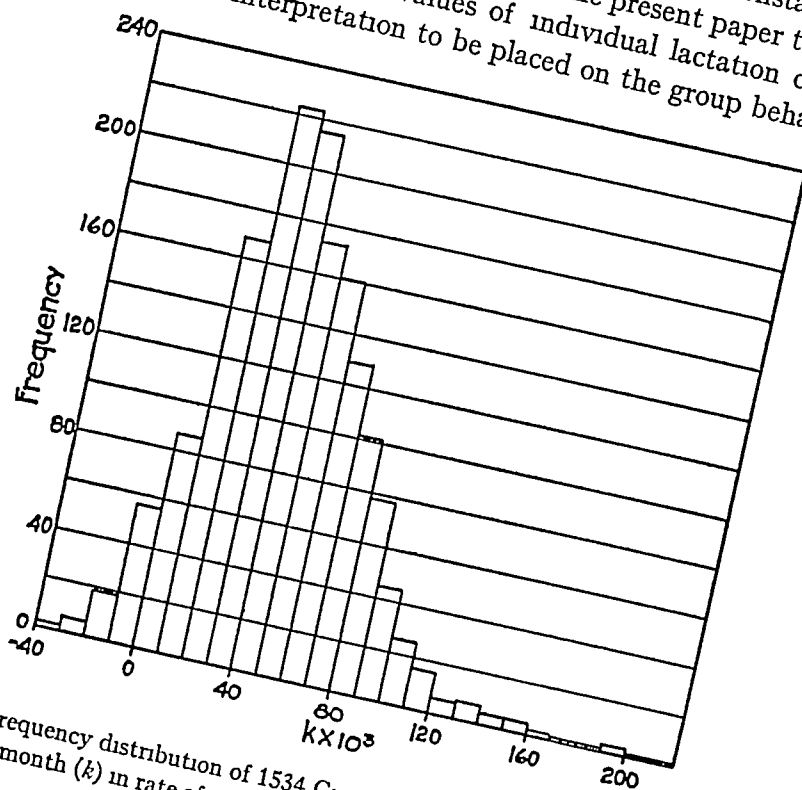
¹ Sturtevant, E. L. *Rep. New York (Genesee) Agric. Exp. Station* 1886 21-23

² Brody, S., Ragsdale, A. C. and Turner, C. W., *J. Gen. Physiol.* 1922-23 v, 441

³ Gaines, W. L. and Davidson, F. A. *J. Gen. Physiol.* 1925-26 ix 325

LACTATION CURVE

curve in simple mathematical terms, the chemical interpretation is open to question. From the viewpoint of Brody *et al* the factor k of the equation corresponds to the specific velocity constant of the chemical reaction. It is the purpose of the present paper to present some results showing the k values of individual lactation curves as bearing on the interpretation to be placed on the group behavior.¹



1 Frequency distribution of 1534 Guernsey records with respect to rate of yield per month (k) in rate of yield

Individual Lactation Curves

ve of the type $\frac{dy}{dt} = Ae^{-kt}$ has been fitted by an adaptation of and previously described to each of 1676 Guernsey records. In on $y = \text{yield}$ and $t = \text{time in months}$. Yield for a month here given are taken from a manuscript presenting a broader in- of the k values, which has been submitted for publication to the Agri- ment Station.

represents the rate of yield at the middle of that month, with an entirely negligible error. Energy value of the milk solids had been used as being the most fundamental of the several available measures of yield. The distribution of the k values of these records, excluding 142 which were highly irregular, are given in Fig 1. For a chemical interpretation of the lactation curve we may consider that the data of Fig 1 represent 1534 determinations of the velocity constant k .

It will be noted from Fig 1 that the k values fall into a quite regular order approaching a normal distribution. The mean of the array is 0.4425 ± 0.0055 , standard deviation, 0.3219 ± 0.0089 , and coefficient of variability, $72.75 \pm .89$. There is thus shown a high degree of variability in the k constants.

Ascending Lactation Curves

It is to be noted further that 83 of the lactation curves, 5.41 per cent of the total, show negative k values, that is, the slope is positive. The velocity constant is not only highly variable, but apparently even reverses its sign in an appreciable number of cases. Obviously some modification of the simple monomolecular interpretation is necessary.

The group behavior of these ascending records is not in good conformity with the equation type, being somewhat aberrant at the start and finish. Indeed, to anyone conversant with the great energy transformations performed by the lactating cow it would be absurd to suppose that the lactation curve could continue to ascend for more than a limited time. The point that any hypothesis must satisfy is that within this group the curve does ascend for 9 or 10 months.

Irregular Lactation Curves

Under this head are to be considered the 142 records not included in Fig 1 and necessary to complete a representative sample of the Guernsey records. The average of this group of irregular records is given in Fig 2 together with 4 individual curves to show the diversity of the records that enter into the average. If the monthly yields are plotted on a logarithmic scale against time on an arithmetic scale

realization of the maximum, but the difficulty lies in accounting for the plus deviations which serve to counterbalance the minus deviations

Factors Affecting the Rate of Decrease

That the k values of the lactation curve equations are greatly affected by conditions of feeding and management of the herd is sufficiently evident from the difference in the performance under advanced registry conditions and conditions of commercial milk production. It may be presumed that the same cows that show under advanced registry a mean k value of .044 would show under the less favorable commercial conditions a mean value of .09 to 1. Obviously nutritional conditions are a powerful factor affecting the rate of decrease.

The k values are also closely related to the initial rate of yield, the correlation between k and A being $r = .535 \pm .012$. On the limiting substance theory the initial quantity of this substance is directly proportional to A . The limiting substance is assumed to disappear in accordance with a monomolecular reaction. The velocity constant of the reaction must be assumed, therefore, to vary directly with the initial amount of the limiting substance, in order to satisfy the observed results. One of the laws of unimolecular reaction is that the velocity constant is the same regardless of the concentration of the reacting substance. Consequently, the observed relation between k and A is in conflict with the theory of a limiting substance and its monomolecular inactivation.

On the other hand, regarded as a nutritional matter and bearing in mind the large energy requirements of lactation, it is not surprising that the rate of decrease should be greater the greater the initial rate of yield.

SUMMARY

The validity of the assumption of a substance determining the rate of milk secretion and undergoing monomolecular destruction, based on group behavior, is questioned on the evidence from a large number of individual lactation curves. It seems probable that the rate of decrease in the rate of milk secretion with advance in lactation is dependent upon factors of a nutritional nature.

RATE OF RESPIRATION AS RELATED TO AGE *

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(Accepted for publication, June 21, 1926)

It is usually stated in the literature that respiration is most rapid in the young and actively growing parts of a plant and decreases with age, and sometimes it is said that respiration conforms to the grand period of growth

Considerable experimental evidence upholds the first statement Bonnier and Mangin¹ studied the respiration of several different plants among them *Econymus japonica* Leaves 1 year old respired more per volume of leaf than leaves produced the year of the experiment Data for other plants were obtained (at different temperatures as far apart as 13 degrees but no corrections were made) M. A. Maige² found that most flowers produce more CO₂ per gm. of green weight in the bud stage than when open but some respire most actively in the open stage. G Maige³ found that the intensity of respiration of floral organs decreased with age except in the pistil which often showed increased respiration as long as it continued to develop Nicolas⁴ found that of two samples of twigs the younger had a higher rate of respiration Studying young and adult leaves he obtained the same results Briggs Kidd and West⁵ state that the evidence available (*Helianthus*) is that the respiration per unit dry weight of the whole plant at constant temperature decreases with age.

* Paper from the Department of Botany of the University of Michigan No 250

¹ Bonnier G and Mangin L., Recherches sur les variations de la respiration avec le developpement des plantes, *Ann sc nat bot*, 1885 ii, series 7 315

² Maige, M A., Recherches sur la respiration de la fleur, *Rev gén bot*, 1907 xix 8

³ Maige G., Recherches sur la respiration des différentes pièces florales *Ann sc nat bot* 1911 xiv series 9 1

⁴ Nicolas, G., The variation in the respiration of plants in proportion to age *Bull soc hist nat Afrique nord* 1910 No 7 109 (*Exp Station Rec* 1912 xxvi 628)

⁵ Briggs G E. Kidd F and West C. A quantitative analysis of plant growth Part II *Ann Applied Biol* 1920-21 vii 202.

On the other hand A. Mayer,⁶ and also Rischaw⁷ claim to have demonstrated that the respiration follows the grand period of growth. These investigators enclosed seedling plants in a respiration chamber, and either by daily measurements of the O_2 intake or of the CO_2 given off arrived at the conclusion that there was a grand cycle of respiration conforming to the grand period of growth. In their experiments the normal photosynthetic activity of the plants was prevented and no account was taken of any changes in weight due to the metabolism of the plants. In other words, their results record the respiratory changes in a growing plant independent of any loss or increase in the weight of the plant during its growing period.

The purpose of the present investigation has been to determine carefully the relation between rate of respiration and age. To accomplish this an effort was made to determine the comparative rate of respiration in successive leaves of several plants. In the plants chosen new leaves continue to appear at the top of the plant until the panicles are formed. Before these are formed, however, some of the older leaves at the base have withered away. The successive leaves of these plants, therefore, constitute an age series. In corn the total leaves may number anywhere from 18 to 24, but at any one time there are usually not more than 8 to 12 leaves. In sorghum the total number of leaves developed is 25 to 30 with 8 to 15 active leaves present at one time. In oats and wheat there are only 4 to 6 leaves at any one time. The number of active leaves present appears to be related to age, varietal differences, and water supply. During a dry season the life cycle of a leaf is shorter than during a wet season.

In determining the rate of respiration, the active leaves of the plants were removed and the total CO_2 given off by each leaf was simultaneously determined, by placing the leaves in a battery of Pettenkofer tubes. Inasmuch as the leaves of a plant were all removed at the same time, placed in their respective respiration chambers at the same time, and the CO_2 given off was determined for the same interval, and further, since temperature and other external conditions were identical, it follows that any differences in the rate of respiration are due to differences in the leaves themselves.

⁶ Mayer, A., Ueber den Verlauf der Atmung beim keimenden Weizen, *Landw. Versuchs-Stationen*, 1875, **xviii**, 245.

⁷ Rischaw, L., Einige Versuche über die Atmung der Pflanzen, *Landw. Versuchs-Stationen*, 1876, **xix**, 321.

The rate of respiration given is the amount of CO_2 given off per gm of dry matter or per gm of green weight for the duration of the experiment. Since there were variations in the duration of the experiments and also in the temperature conditions, the rates of respiration in one experiment cannot, except in a general way, be compared with the rates in another. In general, the duration of an experiment was from 18 to 22 hours.

All possible precautions were taken to insure that the air entering the respiration chambers was free from CO_2 , care was also taken that all the CO_2 was absorbed by the barium hydroxide tubes. The barium hydroxide after having been standardized was kept in containers entirely free from CO_2 .

To illustrate the procedure a typical experiment will be described. A corn plant with ten healthy leaves has been selected in the field. It is brought into the laboratory and the leaves are carefully removed and placed in separate bottles of 500 cc, capacity which are completely covered with black paper. The experiment is arranged as follows. First come 10 CaCl_2 towers filled with pieces of soda lime to absorb the CO_2 from the entering air, following each tower is a bottle containing a solution of BaOH , to indicate whether all the CO_2 has been absorbed, to these bottles are attached the respiration chambers, covered with black paper, which in turn are attached to the Pettekofer tubes, containing 100 to 150 cc. of standardized BaOH (the amount depends on the size of the plant, with large plants more BaOH is used than with small plants), each tube is followed by a bottle containing a solution of BaOH , to insure that all CO_2 has been absorbed by the standardized BaOH , these bottles are connected by Y tubes to a single aspirator, by means of which the air is drawn through the apparatus. The rate and size of the gas bubbles passing through each tube are regulated so as to be the same in all tubes. Before the experiment is started the respiration bottles are thoroughly freed of all CO_2 by running CO_2 -free air through them. The experiment is then run for 22 hours. At that time the BaOH in each tube is titrated with N/10 oxalic acid.

1 cc. of N/10 oxalic acid is equivalent to 0.0022 gm of CO_2 , and from the difference between the original titrations of the standardized BaOH and the titrations at the end of the experiment the amount of CO_2 can readily be calculated.

The amount of CO_2 has been calculated both on the basis of dry and of green weight. The results are similar, and as it is more usual to employ dry weight than green weight, and as the respiration is undoubtedly more closely related to the dry material than to the water of the plant, only the data calculated for dry weight are given. As pointed out before, no two experiments were carried out under identical conditions, nor were the plants themselves identical, some of these experiments were performed during the summer of 1923, others

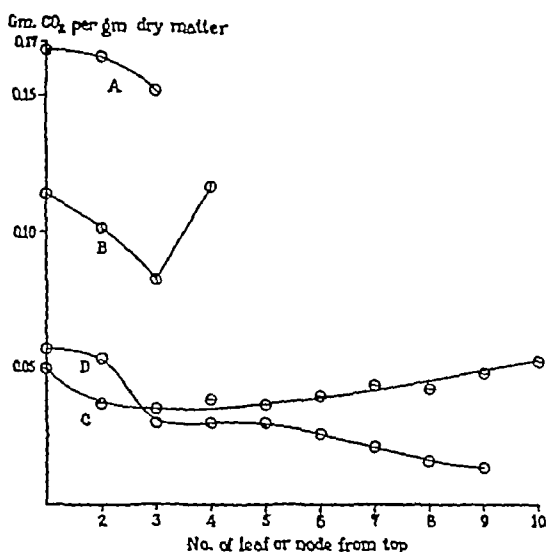


FIG 1 Curve A represents the respiration of 3 corn plants about 4 inches high, with 3 leaves, B, 1 corn plant 6 inches high with 4 leaves, C, 1 corn plant at the time of pollination with 10 leaves. Curve D represents the respiration of a corn stem cut up in such a way that each piece contains one node and part of the contiguous internodes.

during 1924. For this reason no average of the experiments can be obtained. A few of the experiments have been selected as illustrating the condition in these plants.

Corn (*Zea Mays*) was the original plant worked with and the results of a few of the experiments are given in Fig 1. Curves A, B, and C represent plants of different ages with varying number of leaves. Curve A shows that there is a decrease in rate of respiration with age when the plant is very young, while B shows that as the plant ages

and the number of leaves increases the rate of the older leaves increases above that of those slightly younger, and *C* (a plant with ten leaves) shows that the oldest leaves may actually respire more than do the youngest leaves on the plant. Curve *D* represents an experiment with the corn stem, which shows that there is a decrease in respiration with increase in age.

In Fig 2 Curves *A*, *B*, and *C* represent respiration in *Sorghum vulgare*. All of these experiments show that at first there is a decrease in respiration as the leaves age, but that after a certain age has been

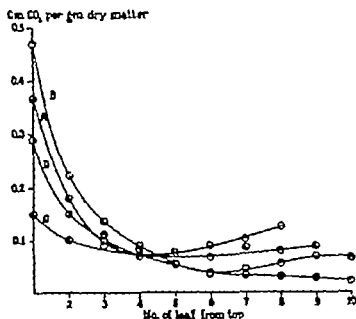


FIG 2. Curve *A* represents the respiration of 4 sorghum plants 1 foot high with 8 leaves. Curve *B* 1 sorghum plant 3 feet high with 10 leaves, *C* 1 sorghum plant developing tassel, with 9 leaves. Curve *D* sunflower plant about half grown with 10 leaves.

reached there is a gradual increase in rate, which in some instances is nearly as great as that in the youngest leaves. Curve *D* represents leaves of sunflower (*Helianthus annuus*), and in this as well as in other experiments on sunflower there is a decrease from the first with no subsequent increase.

Fig 3 represents results obtained with oats (*Avena sativa*). The two experiments were conducted at the same time. These experiments show that the respiration in the oldest leaves is much more rapid than in the youngest.

In Fig 4 are results of two experiments with nearly mature wheat (*Triticum sativum*) These plants had five leaves but the two oldest ones were dying and so cannot be counted Both experiments show that the oldest healthy leaves respired more vigorously than the youngest leaves of the plants

The plants represented in Figs 3 and 4 were nearly mature and the youngest leaves had already reached the age at which the respiration is at its lowest, all the curves but B of Fig 4 show only increase in respiration with age rather than a decrease followed by an increase

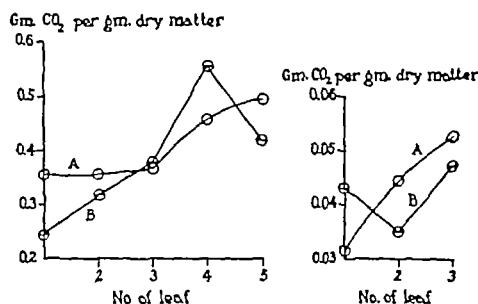


FIG 3

FIG 4

FIG 3 Two oat plants nearly mature The two experiments were conducted at the same time These curves strikingly bring out individual differences

FIG 4 Two wheat plants, nearly mature, each plant had 5 leaves, but the two lowermost were practically dead The two experiments were conducted at the same time They also show individual differences

It is quite apparent from the data given that when dry weight is taken as a criterion for judging the comparative rate of respiration in the various leaves of corn, sorghum, sunflower, oats, and wheat the respiration cycle does not correspond to the grand period of growth, nor is there a decrease in respiration with age except in the leaves of sunflower and corn stem The reason other investigators have not noted this before is that not enough controlled experiments have been performed In most instances a few leaves of one age were taken at one time at a certain temperature and a few leaves of a different age at another time, at a temperature different from the first, without any correction being applied As far as the writers are aware no experiments have been performed in which a series of leaves or other plant

parts, differing in age have been studied at the same time. It is obvious from the figures given in this paper that, if only two leaves had been taken, in practically all instances the older leaves would have respired less than the very youngest on the plant, but it is equally obvious that by taking very young leaves, middle aged and old leaves at the same time, the old leaves are found to respire more rapidly than the middle aged ones, though usually less than the youngest.

The writers are not prepared to say that as protoplasm ages it respire less and that as it gets still older it begins to respire more actively. But it seems that when the amount of CO_2 given off is calculated on the basis of dry weight the rate of respiration increases to some extent after middle age. This is true of the leaves of corn, sorghum, oats, and wheat, but not of sunflower leaves and corn stems.

The amount of CO_2 given off per gm. of dry or of green weight is probably not a good criterion of respiration. That, however, is customary. As a cell increases in size and in age, the total amount of protoplasm probably remains the same while the dry material (cell wall, stored food, etc.) increases. The respiration is presumably connected with the protoplasm. Then if the amount of respiration of an old leaf is calculated on the basis of the total dry weight (cell wall, stored food, etc.) it is obvious that the rate per gm. of dry weight is going to be less than in the young leaves, though the rate per gm. of protoplasm may be the same. The writers have unpublished data to show that as leaves grow older there is an increase in the percentage of dry material up to a certain age, when there is sometimes a decrease. A method which would take into account only the amount of protoplasm would be much more accurate. Perhaps the CO_2 could be calculated on the basis of amino nitrogen or total nitrogen in a plant part.

SUMMARY

In the present paper it is shown that as the leaves of corn, sorghum, wheat, and oats increase in age there is a decrease in rate of respiration, but that as the leaves become still older (past about middle age) the rate gradually increases.

CONCERNING THE INFLUENCE OF POLARIZED LIGHT ON THE GROWTH OF SEEDLINGS

By DAVID I. MACHT

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Dunning, Baltimore)

(Accepted for publication June 11 1926)

The biological effects of polarized light have until a year or two ago, not been the subject of any scientific experimental study. Recently a few contributions on the subject have appeared. Baly and Semmens published a short note describing the stimulating effect of polarized light on the hydrolysis of starch by diastase (1). These observations were confirmed by the present author (2) who at the same time published several short communications on the pharmacological and other biological effects of polarized light (3) and also described in collaboration with Justina Hill some experiments on the growth of yeast and bacteria (4). In this last communication, Miss Hill and the author called attention to the apparent stimulation of bacterial growth by polarized light. Similar observations on bacteria were published independently by Morrison (5), and recently two Indian investigators have also published a short note claiming that the bacteria of typhoid fever and cholera thrive better in polarized light than in non polarized light (6). In the present paper the author proposes to describe a series of experiments on the growth of seedlings in polarized and non polarized light which were begun early in 1924 and continued up to the present time.

Method

The growth of young seedlings of *Lupinus albus* in a nutrient phyto physiological (Shreve's (7)) solution was followed by measuring the elongation of the roots at intervals of 24 hours. The influence of polarized and non polarized light was studied by means of the following apparatus which was designed jointly by the author and Professor

A H Pfund of the Department of Physics of the Johns Hopkins University and was constructed under Professor Pfund's supervision and calibrated by him. A box or cell in the form of a truncated pyramid was constructed 80 cm high, with a lower base 60×45 cm and upper end about 25 cm square, the back of the wall of the cell being perpendicular to the base, and the front wall and *door* being slanting. At the upper or small end of the cell a socket is fixed into which is inserted a large round Mazda tungsten nitrogen electric bulb, of 500 watts power, which serves as a source of light. The lower part of the apparatus or cell is divided into two compartments, completely separated from each other by a blackened partition. The light of the Mazda lamp is allowed to penetrate into the chamber on one side of the apparatus after first passing through a dozen plates of smooth glass, placed at the "polarizing" angle, so that this chamber is illuminated with highly polarized light. The light from the same Mazda lamp, on the other hand, is allowed to penetrate into the second or neighboring chamber after first passing through a pile of smooth plates of glass placed *perpendicular* to the line of propagation of the light, so that this second chamber is illuminated with non-polarized light. The number of glass plates in this second pile was adjusted so that the intensity of the non-polarized light was just equal to the intensity of the polarized light in the first chamber. By boring apertures in the floors of the two respective chambers and taking spectrophotographs of the two transmitted lights, it was found that the spectral range of light waves in the polarizing and non-polarizing chambers was the same, the shortest waves transmitted being about 3650 Ångstrom units. The temperatures in the two chambers were nearly the same, not deviating from each other more than 0.5°C .

The source of light was an electric bulb of 700 candle power. This intensity was of course cut down by passage through the piles of plates, but the intensity of the transmitted light in each chamber was made the same by photometric calibration in the Physics Laboratory, performed by Professor A H Pfund. The intensities in the two chambers were compared by Professor Pfund by reflecting the lights passing through the two sets of glass plates, from a white surface, and allowing the rays to pass through a Lummer tube. The light from the two chambers was thus reflected *diffusely*, and hence was *depolarized*.



FIG. 2



FIG. 1

FIG. 1 Polarizing apparatus Front view with door removed.

FIG. 2 Polarizing apparatus, Side view Right wall removed and showing the chamber illuminated with polarized light

before the comparison was made. Thus while the eye was used in comparing, it could not be argued that there might be a difference in the physiological effects of polarized and non-polarized lights on the eye. Of course such an objection would be purely hypothetical, as, so far as is known, no difference in the effects on the eye between polarized and non-polarized lights has ever been noted, and if such a difference should be experimentally demonstrated, it would be a fundamental physiological discovery.

In order to make sure that small variations in intensity of the control did not affect the results, a number of experiments were made with the non-polarized light of a slightly greater or slightly lesser intensity than the polarized light (by changing the number of plates in the control chamber). Such variations did not appreciably change the marked effect of the polarized light.¹

The temperatures in the two chambers were the same to within a fraction of a degree, as indicated by thermometer readings and also by thermographic tracings. Here again a number of experiments were made, in which the temperature in the control chamber was purposely made a little higher or a little lower than in the polarized chamber, respectively, and the results obtained still showed a definite stimulation of growth produced evidently by polarized light.

RESULTS

Two sets of *Lupinus* seedlings, A and B, generally of 10 each, were carefully measured, then placed in hard glass tubes with Shive's solution, and one set was put in each chamber, Set A in polarized light, and Set B in non-polarized light. The plants were irradiated by the polarized and non-polarized lights, during the daytime, and were left in the dark overnight, when the electric current in the laboratory was turned off. On measuring the growth of the roots of the two sets of plants on the following day it was found that the seedlings exposed to polarized light had grown distinctly more than the other set. The two sets of plants were then interchanged, by placing them in the opposite chambers, that is Set B in polarized light and Set A in non-

¹ In other experiments with polarized light to be published later, intensities were compared by means of a bolometer.

polarized light, and exposed to the two kinds of light again. On the following day, when the growth of the roots was again measured, it was found that the original "non polarized" set (B) which was this time exposed to polarized light actually outgrew the original "polarized" set (A) of plants, which, on this second day, was placed in the non polarizing chamber.

The two sets of plants were again reversed for a second time, and it was found again that the seedlings grew more in polarized light. On reversing the position of the two sets a third time, better growth in polarized light was again observed. Such experiments with *Lupinus* seedlings were made a number of times with the same results. The following protocols will serve as illustrations.

TABLE I.

Set A.					Set B.				
Seed ling No.	Normal length.	Polarized light.	Non- polarized light.	Polarized light.	Seed- ling No.	Normal length.	Non- polarized light.	Polarized light.	Non- polarized light.
	mm.	mm.	mm.	mm.		mm.	mm.	mm.	mm.
1	38	49	60	88	1	37	46	57	68
2	35	46	53	80	2	31	50	69	80
3	31	53	60	82	3	23	46	57	64
4	33	39	48	65	4	31	47	62	70
5	24	49	56	71	5	27	43	62	70
6	24	60	64	70	6	34	52	59	64
7	25	50	62	71	7	27	44	70	75
8	29	51	56	64	8	33	47	48	78
9	34	44	56	77	9	33	50	68	77
10	27	41	49	72	10	30	37	65	69
	300	482	564	740		306	462	617	715
		Incre- ment 182.	Incre- ment 82	Incre- ment 176			Incre- ment 156.	Incre- ment 155	Incre- ment 98

Table I gives the results of an experiment made on 2 sets of plants of 10 seedlings each. Set A was exposed to polarized light on the 1st day, to non polarized light on the 2nd day, and to polarized light again on the 3rd day. In the case of Set B the order of exposure was

reversed, that is on the 1st day the plants were exposed to non-polarized light, on the 2nd day to polarized light, and on the 3rd day again to non-polarized light. The normal or original length of each root is indicated in the first column and the length at the end of each 24 hours in the successive columns. It will be seen that the increment in every case was greater when the seedlings were exposed to polarized

TABLE II *

Set A.						Set B					
No.	Apr 26 Original length	Apr 27 Polarized	Apr 28 Non- polarized	Apr 29 Polarized.	Apr 30 Non polarized.	No.	Apr 26 Original length	Apr 27 Non polarized	Apr 28 Polarized.	Apr 29 Non polarized	Apr 30 Polarized
	mm	mm.	mm	mm	mm		mm	mm	mm	mm	mm
1	47	55	69	81	76	1	33	57	70	82	72
2	42	60	78	74	79	2	32	58	61	68	82
3	47	53	65	87	70	3	36	51	68	70	70
4	38	66	69	71	75	4	45	53	75	78	71
5	48	50	62	76	80	5	42	68	74	79	77
6	32	64	64	77	76	6	40	57	71	81	74
7	39	52	65	77	70	7	45	56	71	72	64
8	45	67	62	77	85	8	36	64	71	76	76
9	37	48	69	80	71	9	49	53	64	69	80
10	35	54	68	68	72	10	42	62	76	73	75
11	36	68	66	72	72	11	48	49	61	77	78
12	42	59	68	73	70	12	44	47	60	78	81
13	37	59	74	74	72	13	33	61	73	77	79
14	51	63	63	77	75	14	48	60	69	75	72
15	42	56	58	73	69	15	45	57	63	75	85
	618	874	1000	1237	1112		618	852	1027	1130	1136
		Incre- ment 256	Incre- ment 382	Incre- ment 619	Incre- ment 494			Incre- ment 234	Incre- ment 409	Incre- ment 512	Incre- ment 518

* In a few cases measurement of seedlings shows a shrinkage on the last day

light and more than that, when the two sets of seedlings were reversed in respect to the form of radiation, the same phenomenon was noted, namely, the greater growth in polarized light.

In the experiments summarized in Table II two other sets of plants consisting of 15 seedlings each were treated as above, only in this experiment the interchange from polarized to non-polarized light and

TABLE III
Experiment 10

Series A ¹			Series B ¹		
May 3 1926.	May 4 1926.	May 5 1926.	May 3, 1926.	May 4 1926.	May 5, 1926.
Normal.	Polarized.	Non-pol arized.	Normal.	Non-pol arized.	Polarized.
50.1 mm.	67.3 mm.	75.4 mm.	51.9 mm.	64.6 mm.	77.4 mm.
Mean temp. 26° C.	Mean temp 26°C.	Mean temp 26°C.	Mean temp 26°C.	Mean temp 26°C.	Mean temp. 26°C.

TABLE IV
Experiment 11

Series A (Covered seeds.)			Series B (Covered seeds.)		
May 3 1926.	May 4 1926.	May 5 1926.	May 3 1926.	May 4 1926.	May 5 1926.
Normal.	Polarized.	Polarized	Normal.	Non-polar ized	Non-polar ized
30 0 mm	35 7 mm.	39 1 mm.	29 6 mm	35 3 mm.	37 8 mm.
Mean temp. 22°C.	Seeds cover ed Mean temp 22°C.	Seeds un- covered Mean temp 22°C.	Mean temp 22°C.	Seeds cover ed. Mean temp. 22°C.	Seeds un- covered. Mean temp 22 C.

TABLE V
Experiment 12

Series A ^a (Covered roots.)			Series B ^a (Covered roots.)		
May 3 1926	May 4 1926.	May 5 1926	May 3 1926	May 4 1926.	May 5, 1926
Normal	Polarised	Non-polar ized	Normal.	Non-polar ized	Polarised
27.2 mm.	36.4 mm.	40.0 mm	26.4 mm	34.2 mm.	41.3 mm.
Mean temp. 22°C.	Mean temp. 22°C	Mean temp 22°C.	Mean temp. 22°C.	Mean temp. 22°C.	Mean temp 22°C

vice versa was made on 4 successive days until the plants were too long to be measured conveniently. Here again it will be seen that in every case greater growth took place in polarized light. (In this table the order of the seedlings is not the same in each column as they were not placed in individual tubes but in flasks containing several seedlings each.)

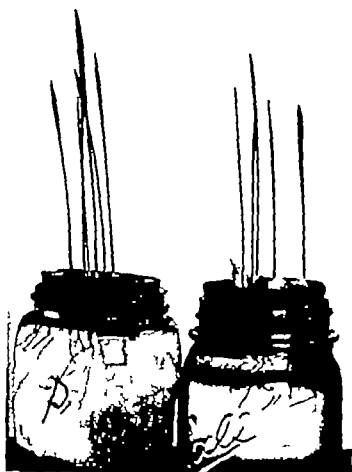
Table III shows an analysis of the above phenomena. In Experiment 10, two sets of plants consisting of 10 seedlings each were placed

TABLE VI
Squash Seedlings

Set A.				Set B			
No	Original length	Polarized 24 hrs	Polarized 72 hrs	No	Original length	Non-polarized 24 hrs	Non polarized 72 hrs
	mm	mm	mm		mm	mm	mm
1	47	58	69	1	45	55	57
2	36	47	54	2	34	37	47
3	32	44	58	3	32	34	47
4	21	27	46	4	22	25	31
5	41	51	71	5	43	54	45
6	31	40	63	6	33	44	80
7	42	47	56	7	37	40	83
8	21	29	52	8	22	35	63
9	19	28	66	9	21	26	34
10	16	26	55	10	18	30	38(?)
	306	397	590		307	380	525
		Increment 91	Increment 284			Increment 73	Increment 218

in polarized and non-polarized light, the figures here given indicating the sum of the root lengths. It will be noted here again that each set of plants grew better in polarized light. Experiment 11 illustrates the effect of wrapping the seeds or cotyledonous portion of the plants with tinfoil. It will be noted that when the beans were wrapped in tinfoil and thus protected from light altogether, growth was exactly the same in both chambers. When, however, these wrappings were removed on May 5th, better growth occurred in polarized light than in non-polarized light. Experiment 12 shows the results obtained

when the stems and roots of the seeds were protected from the light but the seed portion was left uncovered. Here better growth took place in polarized light than in non polarized light thus indicating that the stimulation of growth by polarized light is due to photochemical changes induced in the seed portion. This, of course, harmonized prettily with the findings of Baly and Semmens and the



Polarized.

Non-polarized

FIG 3 Wheat seedlings

present author in connection with the effect of polarized light on the diastatic hydrolysis of starch

The author performed most of his experiments on seedlings of *Lupinus albus*. A number of experiments, however, were also performed on other plants. In Table VI are the results obtained with squash seedlings. Here again it will be noted that better growth

occurred in polarized light than in non-polarized light. A number of experiments were made with wheat seedlings, as these seedlings grow several roots which are difficult to measure, the growth was studied by measuring the elongation of the stems and leaves. In Table VII and Fig 3 are shown the results obtained in one such experiment. Two sets of 6 seedlings each were exposed to polarized light and non-polarized light and the length from the root to the tip of the blade of

TABLE VII
Wheat Seedlings
(See Fig 3)

Set A.	Set B
In polarized light	In non polarized light
6 seedlings.	6 seedlings
Original length from roots to tip of blade, 402 mm	Original length from roots to tip of blade 406 mm
3 days later, 656 mm	3 days later, 588 mm
4 " " 921 "	4 " " 804 "

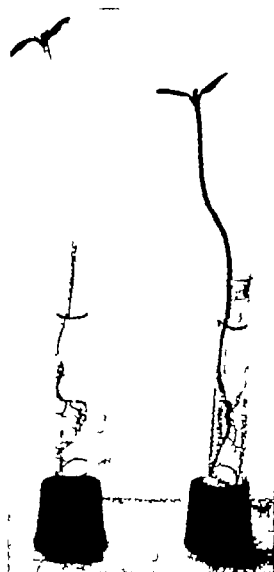
TABLE VIII
Helianthus (Sunflower)
(See Fig 4)

Seedling A.	Seedling B
Dec 16, 1925 Length of stem, 120 mm Placed in polarized light	Dec 16, 1925 Length of stem, 120 mm Placed in non polarized light
Dec 18, 1925 Length of stem, 140 mm	Dec 18, 1925 Length of stem, 125 mm
" 19, " " " 147 "	" 19, " " " 127 "

each seedling was measured. It will be noted that better growth took place in polarized light. In several other experiments with wheat seedlings the growth of the plants was studied by weighing them. In this way it was also found that the seedlings exposed to polarized light weighed distinctly more than those exposed to non-polarized light. In Table VIII and Fig 4 is shown the effect of polarized light on two seedlings of the sunflower, the length of the stems was measured and it will be noted better growth took place in the polarizing chamber.

SUMMARY

While these experiments are not exhaustive a sufficient number have been made to warrant the statement that the effect of polarized light of the visible spectrum on the growth of various seedlings and



Polarized.

Non polarized.

FIG 4 Sunflower seedlings

more particularly on the growth of *Lupinus albus* is somewhat different from that of non polarized light. This is especially convincing in view of the results obtained with double sets of plants which were alternately exposed to polarized and non polarized lights of the same

intensities and at the same temperature. In every experiment thus performed the set which was placed in a polarizing chamber grew better. It is, furthermore, interesting to note that the phenomenon above observed did not take place when the seed portion of the plants was protected from light by wrapping with tinfoil. This agrees well with previous findings concerning the action of diastase on starch in polarized light. The above researches will be continued on a more elaborate scale but the results so far obtained are deemed worthy of publication in the form of a preliminary communication at the present time.

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ON CURVES OF GROWTH, ESPECIALLY IN RELATION TO TEMPERATURE

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I.

Growth generally manifests an accelerated velocity during the mid portion of a developmental cycle, so that the curve of bulk with time is sigmoid. This fact gave rise (Ostwald, 1902, Errera, 1899-1900, Robertson, 1907-08) to the view that the accumulation of material during growth may be described by the equation for a first-order process in which one of the products of the transformation acts as catalyst. This conception has had a vigorous enlargement (Robertson, 1923), looking toward the description of the velocity of development as governed by a succession of first-order chemical processes, "master reactions," which are self-accelerated.

Several difficulties are connected with the application of this idea. One of these is that in fitting simple logistic (autocatalytic) curves to actual data, it has usually been assumed (*cf.* Robertson, 1923, 1925-26) that the curve of growth in any cycle is symmetrical about a mid point of inflection. This follows from the nature of the equation commonly taken to give the course of such an autocatalyzed process

$$\frac{dx}{dt} = K x (A - x) \quad (1)$$

where A signifies the initial endowment of the growth promoting precursor, x the amount formed after time t . The point of inflection in the integral curve of this equation is located at

$$x = A/2.$$

A method of testing the sufficiency of this form of the autocatalysis equation is afforded by the study of rates of development as controlled

by temperature. The autocatalytic relationship (Lotka, 1925) is of so very general a character, arising in any kind of a situation where a limited progress is facilitated by the conditions created through its initiation¹ but progressively inhibited by proportionately enforced exhaustion, that some test of this sort is necessary if the form of growth curves is to be satisfactorily understood. Thus if the simple logistic were an adequate description of growth velocity, temporary alteration of the temperature of the development would not be expected to modify the temperature characteristic (Crozier, 1924-25, *a*) for the remainder of the development. Since the temperature characteristic for the velocity constant K must be constant, changing the temperature therefore merely multiplies the time coordinates of the growth curve by a constant. The temperature characteristics obtained from what may be termed "partial developmental periods" should be the same as for the total developmental interval (within one cycle). There is adequate indication (Bliss, 1925-26) that this may not be the case. Consideration of this fact results in a modification of the formulation of the "autocatalytic" curve of growth. The modification has the merit of greater consonance with chemical theory, and of indicating a direct interpretation of the sort of results to be expected when the temperature is changed during the course of a developmental cycle. It may also give some light upon the nature of temperature characteristics for development at constant temperatures.

Let it be supposed that at the beginning of a developmental cycle there is available an unrenovable quantity, A , of a substance giving rise to another, a , which determines the velocity of growth. We are especially interested in "velocities of growth" as measured by the reciprocals of the times required to attain a given stage of development. We will suppose that the material A gives rise to a by a first order reaction, and that a serves as catalyst for this change. The reaction $A \rightarrow a$ will therefore be governed by a velocity constant (K_1) proper

¹ An interesting instance is given by the growth of knowledge of the variety of the amino acids, as plotted by Cohn (1925). A still different type of S-shaped curve may result from estimations of growth in which the numbers of cells of colonial protozoans are counted (Fauré-Fremiet, 1922), in certain species the mode of dichotomy results in the curve for number of individuals against time appearing "autocatalytic."

to it in the absence of the influence of x , and also by the velocity constant due to catalysis by x . The decomposition of A must therefore be conceived as made up of two parallel reactions, and its differential equation is then

$$\frac{dx}{dt} = (K_1 + K_2 x) (A - x) \quad (2)$$

where K_2 is the velocity constant associated with x as catalyst

The velocity of formation of x will pass through a maximum when

$$x = \frac{K_1 A - K_2}{2 K_2} \quad (3)$$

Therefore, if any change of condition, such as temperature, influences K_2 and K_1 unequally, the form of the curve connecting x with time will be changed and the point of inflection will move to a new relative position. Thus when K_2 is made relatively smaller, the inflection point occurs earlier (assuming A the same), and the shape of the curve is significantly altered.²

It is to be noticed that in such a system the point of inflection is found at $x = A/2$ when K_1 is of inappreciable magnitude, whereas, in case K_2 is very small the equation approaches that of the usual monomolecular curve without detectable autocatalysis.

II

Integration of (2) yields

$$t = \frac{1}{K_1 + K_2 A} \ln \frac{A (K_2 x + K_1)}{K_1 (A - x)} \quad (3)$$

The curve of this equation may be applied to various series of observa-

² This formulation seems simpler and leads to more suggestive consequences so far as concerns the planning of experiments, than does Robertson's (1923) suggestion of the pseudo-reversible character of the 'master process' in growth; moreover it would appear to be the obviously correct equation for an autocatalytic system. Robertson pointed out that if a reverse process in the growth equation is of higher order than the direct, the curve of increasing bulk with time becomes asymmetric about the inflection point; many if not most growth-curves are in fact asymmetric (*cf.* Brody 1925-26). A different mode of dealing with the asymmetry has more recently been employed by Robertson (1925-26) which amounts to assuming that x has a positive value at the beginning of a cycle.

tions, for example, to the growth in height of sunflower plants (data of Reed and Holland, 1919) The fit (Fig 1) may be made distinctly better than when the "simple" curve of autocatalysis is employed An adequate test of the descriptive validity of this equation for growth might be sought in the curve of increasing weight for a mammal, where temperature variation may be neglected Donaldson's data on

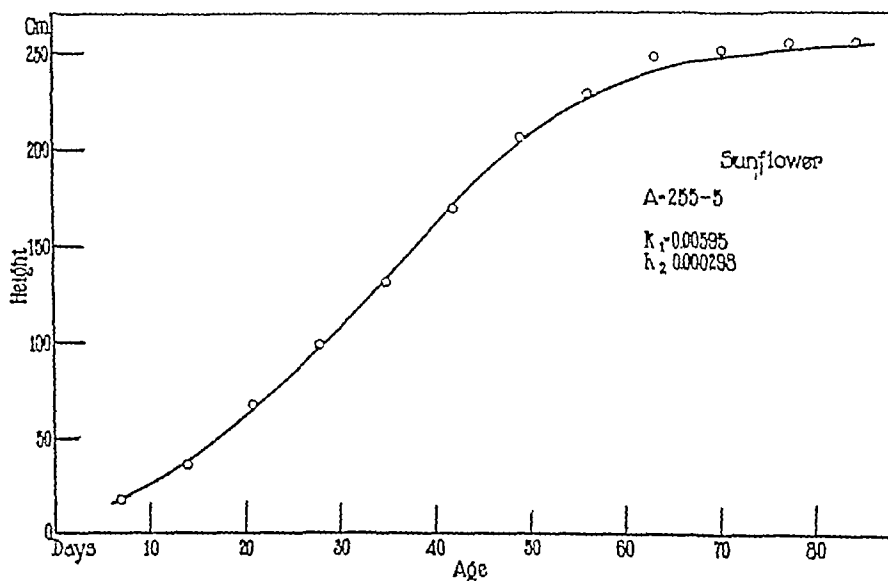


FIG 1 Growth in height of the sunflower, data of Reed and Holland (1919), the curve is that of the equation

$$t = \frac{1}{k_1 + k_2 A} \ln \frac{A (k_2 x + k_1)}{k_1 (A - x)},$$

the cycle starting at $x = 5$ cm, with $k_1 = 0.00595$, $k_2 = 0.000298$, A , the mature height, is taken = 250

the growth of the rat (Donaldson, 1915) are suitable for such a test, though undoubtedly complicated by the cyclic character of growth in mammals (Donaldson, 1906, Robertson, 1907-08, Brody and Ragsdale, 1922-23) Robertson (1923) has fitted to this data two "fused" logistic cycles, but the agreement is not especially good In Fig 2 it is shown that these data are fitted sufficiently well by the autocatalytic equation in the form $dv/dt = (K_1 + K_2 x) (A - x)$, except that for a

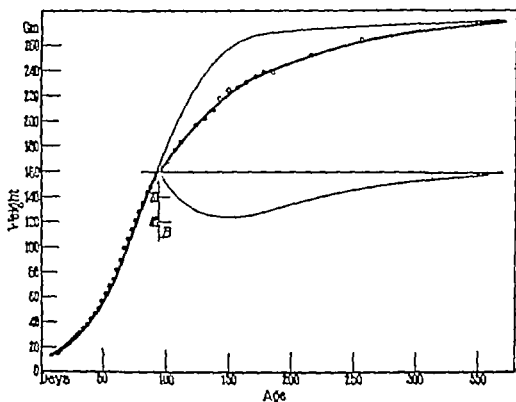


FIG. 2. Data on the growth of unmated male white rats (Donaldson, 1915) adhere fairly well to the equation

$$t = \frac{1}{k_1 + k_2 A} \ln \frac{A (k_2 x + k_1)}{k_1 (A - x)}$$

for the first 100 days. It is assumed that the weight reflects the amount of a growth-determining material x produced in a reaction $A \rightarrow x$, which is catalyzed by x that the value of A is given by the maximum weight (for convenience 280 gm.), and that the cycle starts at $x = 10$ gm. The constants for the curve as drawn are $k_1 = 0.00135$, $k_2 = 0.000135$, $x = \text{weight} - 10$.

Beyond 95 days age, it may be supposed that the material x is inhibited by another, B produced in a system of the type $M \xrightarrow{K_1} B \xrightarrow{K_2} N$ where M is unrenewable. The thin inverted curve is the curve of this process with $M_0 = 121$, $k_3 = 0.01663$, $k_4 = 0.02375$, the equation is

$$B = M_0 \frac{k_3}{k_4 - k_3} (e^{-k_3 t'} - e^{-k_4 t'}),$$

t' being counted from $t = 95$.

The curve drawn through the observed weights (circles) is obtained by subtracting B from x .

period after 100 days age the deviation is great. It is an interesting fact, which may perhaps be used to obtain another view of the mechanism of growth "cycles," that the deviation after 100 days from the formula fitting the earlier course of the data and also its terminal range, may be accounted for quantitatively and exactly by assuming that beyond age 95 days the growth-controlling substance, x , is inhibited (but not destroyed) in proportion to the amount of a substance B produced in a system of the type $A \xrightarrow{K_3} B \xrightarrow{K_4} C$. Assuming suitable values of A , K_3 , and K_4 as found from the deviations of the data from the calculated autocatalytic curve, the accessory curve in Fig. 2 gives the values of B to which the deficiency of x is assumed to correspond. The curve drawn through the plotted points is obtained by taking the difference between B and the calculated x . From this standpoint it may be legitimate to regard the decrease in growth velocity between two cyclic accelerations as due to the presence of an inhibitor, which disappears with time and does not destroy x . It should be possible to relate an inhibition of bulk-increase to known physiological events in the organism dealt with. For the rat it can be pointed out that the maximum in the "correction curve" (B) occurs at an age of 150 days, at which time (Donaldson, 1924) the activity of the thyroid seems to attain a definite maximum and then to decline. This implies that thyroid activity is to be taken as hindering growth in bulk (at this age). Miss King's measurements of growth of rats (Donaldson, 1924) may be fitted in a similar way, with the maximum of the "correcting curve" at very nearly the same age. There is indication that the peculiar growth curve of man (summary of data in Davenport, 1926) may be accounted for in a similar way, with the maximum growth inhibition (male) at about 14 years, but the deviation from calculated autocatalytic curves is so extensive as to make difficult the adjustment of a "correction curve."

III

The way in which the curve of equation (3) may be modified by changing the temperature is illustrated in Figs. 3 and 4. We may assume that for the completion of a developmental stage, say an instar or other definite interval in the differentiation of an insect, there must occur the production of a definite amount of a substance,

x , arising in a first order reaction $A \rightarrow x$, and that x is a catalyst for the reaction. We must suppose that in general the temperature characteristic for the catalytic effect of x will not be the same as that for the catalytic influence promoting the reaction apart from the action of x . As already pointed out, this will so affect the shape of the curve that the graphs at two temperatures are not superimposable. If one half of the developmental period under consideration were to be passed at $t^\circ\text{C}$, this would not mean that one-half of the development would be completed, since the curve is not symmetrical. Then on passing to

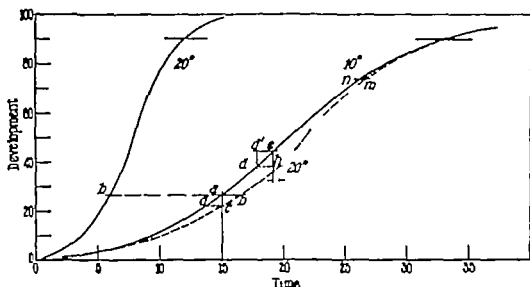


FIG 3 Curves of the equation

$$t = \frac{1}{k_1 + k_2 A} \ln \frac{A(k_2 x + k_1)}{k_1(A - x)}$$

with $A = 100$ in each case. (Time is in arbitrary units)

At 20° k_1 is assumed = 0.010

k_2 " " = 0.005

At 10° , k_1 " " = 0.005 (i.e. $Q_{10} = 2$)

k_2 " " = 0.0017 (i.e. $Q_{10} = 2.94$)

These curves are not superimposable: the dashed curve is that for the 20° conditions expanded by multiplication of the abscissae by a factor which makes the curves coincident at $x = 0.90A$.

If such curves are assumed to underlie development, and if we assume that at completion of a given stage $x =$ a definite fraction of A say = 90 per cent, then Q_{10} for velocity of development is (from the curves) $32.92 \div 12.02 = 2.74$. As A is assumed larger and larger the Q_{10} ratio for velocity of development approaches nearer and nearer to the Q_{10} for k_2 . (If the assignment of Q_{10} 's is reversed Q_{10} for $x = 0.90A$ is 2.13.) Additional details are discussed in the text.

another temperature, the development still to be completed would be (depending on the alteration in the ratio K_1/K_2) either more or less than the fraction uncompleted before the transfer, and the time required to produce the necessary fixed amount of x would be correspondingly greater or smaller than what would be calculated, hence the apparent temperature coefficient for the velocity of the partial de-

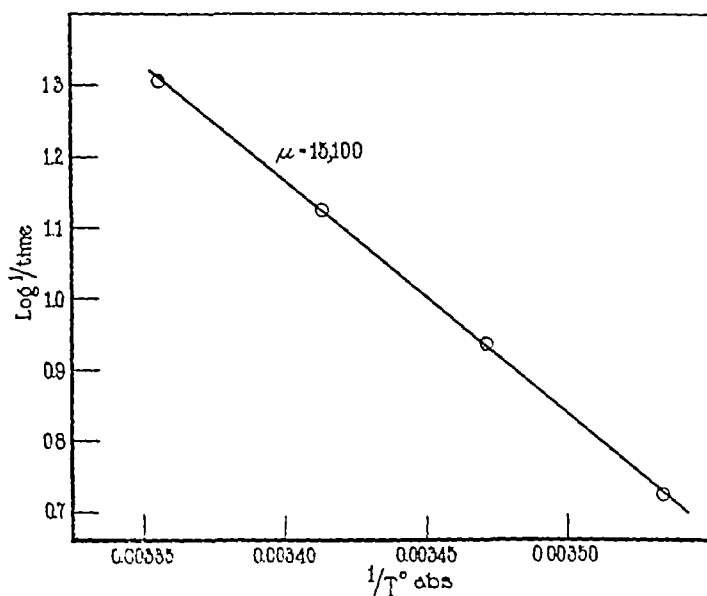


FIG 4 From the autocatalytic curve, with assumed values $A = 500$, $k_1 = 0.08$, $k_2 = 0.0015$, and with μ for $k_1 = 11,000$, for $k_2 = 16,000$, values of the time are calculated to give $\lambda = 490$. The reciprocals of these times are plotted logarithmically against $1/T^{\circ} \text{ abs}$, at 10° , 15° , 20° , 25°C . The relationship is almost exactly rectilinear, and the temperature characteristic obtained from the graph (fitted line) is $\mu = 15,100$. This shows that in a system of the sort discussed in the text it is possible to estimate an apparent critical increment which agrees fairly closely with that for one of two catalytic velocity constants concerned in an autocatalytic activity.

velopment would not be the same as for the total development when passed at constant temperatures. (No account is here taken of differences produced by "breaks" in the curve of developmental rate with temperature.)

The assumption of a definite amount of v as marking the termina-

tion of a developmental interval is required because the end product of growth is the same at each temperature. It is convenient to regard this amount as a fraction of A , the original endowment. With arthropods in particular, the succession of developmental intervals is so definite that one is forced to think of their termination as controlled by "trigger effects," such as might be provided through the accumulation of a governing substance to a critical concentration rather than by the asymptotic approach to completion which would correspond to the extinction of A in our model.

It is desirable to show that deviations in apparent temperature coefficient when part of a developmental period is passed at another temperature, such as those recorded by Bliss (1925-26), may be systematically accounted for. (1) If, in Fig 3, the organism is maintained at 10° for a calculated fraction of the developmental time interval (a to c) it is then ahead of schedule by comparison with the curve of development at 20° (b'), hence the supposed portion of the development still to be completed (from b') is actually less on transfer to 20° , and the apparent temperature coefficient in consequence is larger than if rates of complete development at 10° and at 20° are compared.

(2) On the other hand, if exposure to low temperature occurs during an intermediate fraction (from c to e), the development is "behind schedule" at c (by the amount c to a corresponding to the time c to d), and on arrival at time e , calculated to give a certain fraction of the total developmental time, this will actually be fallen short of so far as concerns differentiation by the amount h , so that the degree of development is then in fact that represented at point d' . If now the organism be brought back to 20° , the developmental level is indicated at point h , which is ahead of that assumed at time e during subsequent development at 20° the developmental course still to be completed is thus less than that calculated and hence appears faster, but with the conditions shown the apparent acceleration is less than in the first case, where transfer from the 10° to the 20° curve results in a considerable time-saving and therefore in a marked increase of calculated temperature coefficient.

(3) Again, if transfer from the 20° curve to the 10° be made late in development, as at point m , time is lost, because the relative develop-

ment is greater at the same time along the 10° curve, therefore the calculated temperature coefficient is actually less than that obtained from uninterrupted developments at 10° and at 20°

For the prepupal period of *Drosophila*, with a normal temperature characteristic 16,850, Bliss (1925-26) found that in experiments of type (1) the apparent μ was 20,220, of type (2), 18,770, of type (3), 16,570. There is additional indication of just this sort of relationship in data given by Titschak (1926) for the rate of development in the clothes moth *Tineola*. The particular curves drawn in Fig 3 are of course intended merely to show that it is possible to explain such results. It is significant that this explanation turns upon properties of an equation which describes with some precision the time course of growth and differentiation such as can be visibly evidenced by increasing weight, and which it is therefore legitimate to suppose may describe even more accurately the progress of developmental changes measurable only by the incidence of their end-results

IV

Although few investigations of growth permit very precise estimations of critical thermal increments, it is nevertheless important that there is indication of diverse magnitudes of μ , the temperature characteristic, for different cases, and that these values are suggestively close to those known to be associated with various other vital processes (Crozier, 1925-26). A careful investigation of a particular developmental stage in *Drosophila* has been made by Bliss (1925-26), Brown (1926-27) has determined the temperature characteristics for a developmental interval in cladocerans, the values of μ derived for these phenomena correspond to values repeatedly found in processes of quite different sorts (Crozier, 1925-26, Crozier and Stier, 1925-26). This is a striking fact, because one would be inclined to believe that chemical mechanisms controlling growth might be very different from those having to do, for example, with the regulation of the heart-beat or of breathing movements. This suspected difficulty might be overcome if it could be shown that the velocity of development, or the duration of life in particular stages, is determined, not by the magnitude of some simple underlying chemical change, but by the velocity with which this change is taking place. Northrop (1925-26) has indeed

shown that the duration of life in *Drosophila* does not depend upon the transformation of a definite amount of energy (Rubner), since the amount of CO_2 produced during life is not a measure of the life duration

Aside from this possibility, however, the curve of equation (3) has some interesting properties which indicate another mode of interpretation. The reciprocal of the time for production of, say, $x = 0.90 A$, will have a temperature coefficient which depends on the magnitudes of K_1 , K_2 , and A , and of the temperature coefficients of K_1 and K_2 . If A be put = 100, and $K_1 = 0.010$, $K_2 = 0.005$, at 20° , with the respective temperature coefficients Q (10° - 20°) = 2.0 and 2.94, then Q (10° - 20°) for $1/t$ will be 2.74. Thus the temperature coefficient for $1/t$ may agree very closely with that for one of the two velocity constants.

This kind of relationship may be illustrated by one particular set of assumptions as to the values of A , K_1 , and K_2 , when it is supposed that $\mu = 11,300$ for K_1 and $\mu = 16,500$ for K_2 . We desire to see whether the values of $\log 1/t$ will in such a case give a rectilinear relationship to the reciprocal of the absolute temperature, as seems to be the case in growth. It is seen that with very small deviation, such as would probably be overlooked in practical cases, they, indeed, do give this relationship (Fig. 4).³ This is sufficient to demonstrate that an autocatalytic system in which two velocity constants are implicated may permit the approximate evaluation of temperature characteristics, and that these may even be quite close to those corresponding to the

³ It is easily seen that with other values of K_1 , K_2 , the agreement could be very much closer. The values used in this illustration are chosen merely to show the slightly curvilinear character of the plot (Fig. 4) which would otherwise be detected with difficulty.

It may be pointed out that calculations of $1/\text{time for completion}$ of a stage of development based upon the integral form of $dx/dt = (K_1 + K_2 x)(A - x)$ do not yield breaks in the curve of $\log(1/\text{time})$ vs $1/T^\circ \text{ abs}$, even when such temperature characteristics are assumed as reverse the sign of the difference between K_1 and K_2 at the extremes of temperature. In such a case the μ calculated from the times required to produce $x = 0.90 A$ agrees rather closely with that μ , whether of K_1 or K_2 which happens to be smaller. It is of interest that in a system of this kind it is the magnitude of the temperature characteristic, rather than of the velocity constant, which chiefly determines the apparent μ of the resultant

action of specific catalysts. The calculated curve is not exactly rectilinear, but in practice it may be very difficult to decide the origin of deviations at the extremes of the temperature range. It may be pointed out, however, that one may, from this standpoint, expect to find evidence of (1) slight differences in critical increments when contrasting growth phenomena with other common activities, (2) relationships between $\log 1/\text{time}$ and $1/T^\circ$ abs which are not exactly rectilinear, but concave toward the $1/T^\circ$ axis, and (3) deviations of critical increment when development is passed in part at one temperature, in part at another. The testing of these, especially (1) and (2), is at present handicapped by absence of precise data.

v

In discussing growth rates as controlled by temperature it must be kept in mind that the momentary growth rate may vary with time. This may result not merely from the form of the growth-curve, but also because the growth-curve may smooth out recurrent cyclic variations in rate. An instance has already been given (Crozier, 1924-25, *b*), derived from Leitch's measurements of root elongation in the sweet pea. The temperature characteristics in this case differ markedly, depending upon the time period (0.5 hour or 24 hours) involved in the measurement. This is probably related to the fact that cell division occurs at a fairly definite point in a diurnal growth-rhythm (Stålfelt, 1921). Other kinds of complexity are not unexpected, for no one in his senses regards growth as an uncomplicated process. Lehenbauer's (1914) data show that the mean hourly elongation of maize seedlings increases abruptly at 20° , and with time. The average μ above 20° is about 15,500. Where such "breaks" occur in the temperature graph the effects to be expected when time is a significant variable are difficult to predict.

It is, nevertheless, of interest to examine the available measurements of growth to see if they yield anything in the nature of consistent temperature characteristics. A number of observers have studied the growth of fungal colonies as related to temperature. The data are not always presented in the most directly usable form, nor can the probable retarding effects of changes in the medium be discounted with any sureness. In the growth of such colonies on agar plates it is to be

supposed that in the absence of retarding or accelerating effects the rate of enlargement should be constant. A correct measure of growth, assuming only extension in area to occur, would be given by the increase in area per unit of circumference per unit time. When growth of such a colony gives a sigmoid curve with time it is certainly inap-

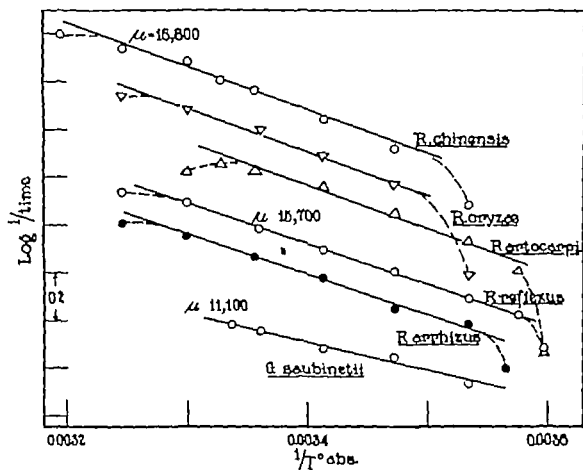


FIG. 5. The velocity of early growth in 5 species of *Rhizopus*, measured by the time required for the germinating hypha to become as long as the diameter of the spore (data from Weimer and Harter, 1923), and the growth of *Gibberella saubinetii* taken as $1 +$ time for colony to attain a diameter of 2.5 cm. (by interpolation from data of MacInnes and Fogelman, 1923). It is apparent that aside from terminal deviations such as are usually encountered the temperature characteristics closely resemble those calculated from data on respiration (Crozier 1924-25 b).

appropriate to regard the curve as describing an inner autocatalyzed growth-controlling process, and hence probably useless to attempt analysis of its precise relations to temperature. Fawcett (1921) records the growth in diameter of colonies of several fungi. These figures have been used to calculate the areal increase per unit time

(24 hours) per unit of growing edge at successive intervals. The rate of enlargement so computed changes with time, in such a way as to suggest that more extensive measurements would, at each temperature, pass through a maximum. The initial growth of the colony so affects the medium that growth is accelerated. Estimations of the temperature characteristic of the inner growth-promoting process would, therefore, require the separation of the effects of temperature upon growth from those involving changes induced in the medium.

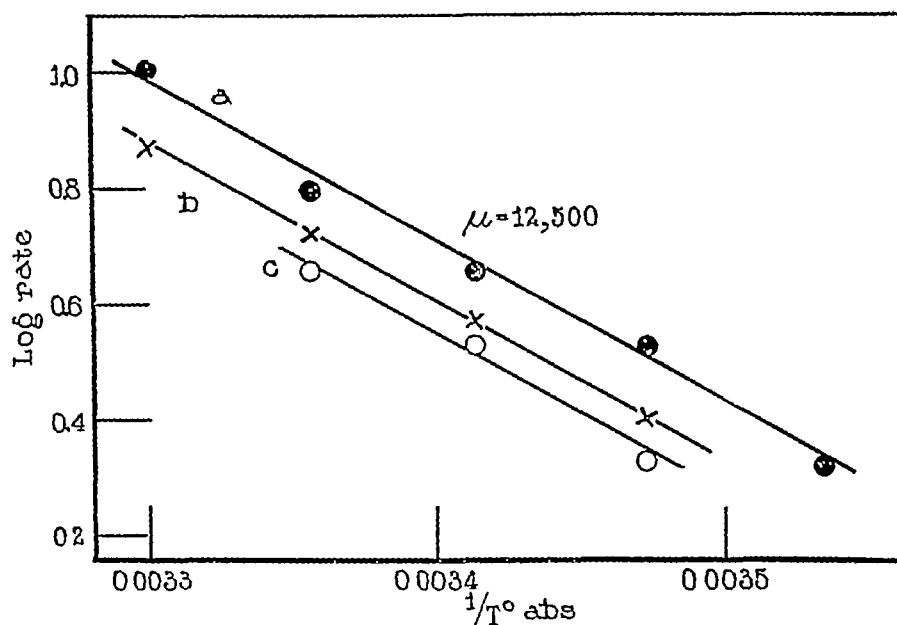


FIG. 6 The rate of decay of sweet potatoes inoculated with *Rhizopus*. The temperature characteristic is sensibly independent of the end-point chosen (*i.e.*, the amount of destruction, presumably determined in greater part by the activity of the fungus). Data from Lauritzen and Harter (1925).

Weimer and Harter (1923) studied the germination and growth of a number of species of *Rhizopus*. From their curves it is possible to plot the reciprocals of the times required for the germ tubes to grow until the length equals the diameter of the spores; these figures provide mean velocities of growth for the attainment of a constant amount of growth (Fig. 5). The agreement with the Arrhenius formula is usually excellent, since the deviations at high and at low temperatures

are no different from those generally encountered in other vital systems. The values of μ accord sufficiently well with those known in other growth phenomena

In such a case it is to be presumed that the temperature characteristic obtained is not a property of the constants in an autocatalytic system, but pertains merely to a metabolic mechanism immediately responsible for increase in bulk

MacInnes and Fogelman (1923) measured the growth of colonies of *Gibberella saubinetii*, and from their figures it is possible to obtain by interpolation the time required at several temperatures to form a colony of given size (diameter). Data from Lauritzen and Harter (1925) may be used in a similar way, giving the time required for *Rhizopus* to produce a given amount of decay in sweet potato. These measurements are plotted in Figs 5 and 6, where the corresponding temperature characteristics are indicated

These instances illustrate the applicability of the Arrhenius equation but they do not permit critical examination of the meaning of the corresponding temperature characteristics. It can be said merely, that in general the critical increments resemble in their magnitudes those already encountered in various other vital activities. A similar vagueness of interpretation is necessary in connection with studies of growth and regeneration in animals. Moreover, and aside from experimental difficulties in temperature control, many cases are disturbed by the fact that the developmental process under examination was not exactly begun at the temperature indicated, but the lot of organisms was distributed to thermostats some little time *after* development had begun. Bearing this difficulty in mind, we may, however, examine the velocity of development in several instances. It should be noted that there is direct justification for regarding a morphological end point as a legitimate end point for our purpose, provided it be timed with precision, Terroine and his associates (Terroine, Bonnet, and Joessel, 1924, Barthélemy and Bonnet, 1924) have indicated that thermal acceleration of development to a constant stage (e.g. to absorption of external gills, in the tadpole, germination of seeds) does not modify the utilization of energy during the growth process,—in other words, the chemical 'balance' is the same at the same stage of development. But in many instances it may be impracticable to obtain a significant morphological end point

The speed of early cleavage of the fertilized ovum has been measured by Loeb and Wasteneys (1911) and by Loeb and Chamberlain (1915), for the egg of *Arbacia*. These two series of measurements are concordant, and exhibit critical increments 12,400, 21,000, 41,000,

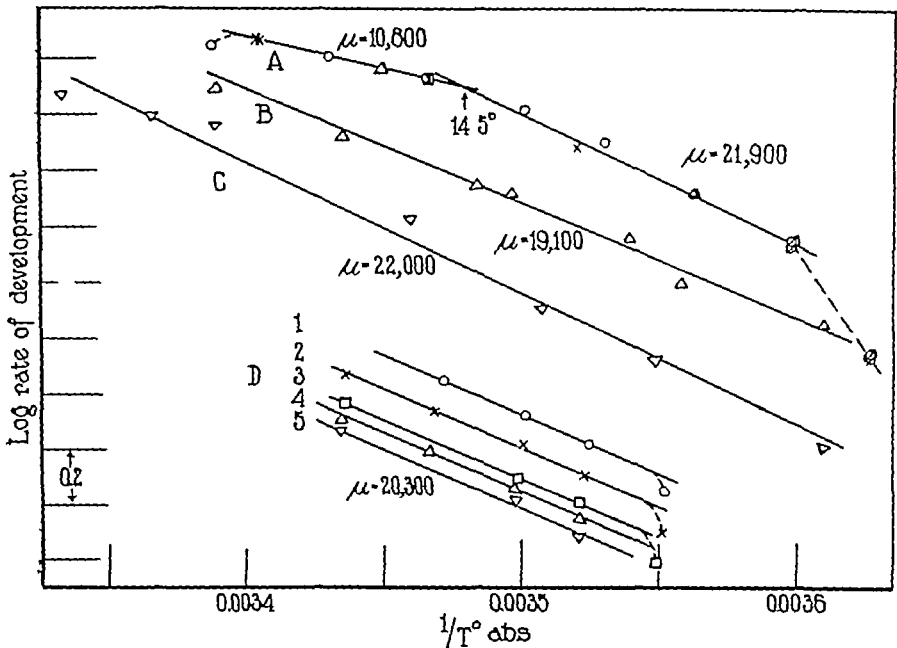


FIG 7 Data upon the rate of cleavage and upon early stages of development in anurans

A Processes involved in the first cleavage, *Rana* (from Krogh, 1914)

B From first cleavage to disappearance of yolk-plug (from Lillie and Knowlton, 1897)

C From first, second, or third cleavage to disappearance of yolk-plug (Lillie and Knowlton, 1897)

D From fertilization to (1) medullary groove, (2) external gills, (3) 3 gill plumes, (4) a length of 7.0 mm, (5) a length of 7.8 mm, *Rana* (Data from Krogh, 1914)

It is clear that there is a measure of consistency in the occurrence of $\mu = 20,000 \pm$. This is indicated also in some fragmentary data from Barthélemy and Bonnet (1924). Hertwig's data (cf Cohen, 1901) also show parallelism in the effect of temperature at different stages of development, but indicate for the intermediate range of temperatures a distinctly lower μ , $17,000 \pm$. Estimations of the rate of growth of the body, and of the tail, in *Rana* and *Bufo*, agree in indicating $\mu = 24,000 \pm$ (data from Lillie and Knowlton, 1897)

with "breaks" at 11° and at 20° (Crozier, 1924-25, b) The velocity of segmentation in eggs of the frog (first cleavage) is plotted in Fig 7, from data by Krogh (1914) The velocity of subsequent early

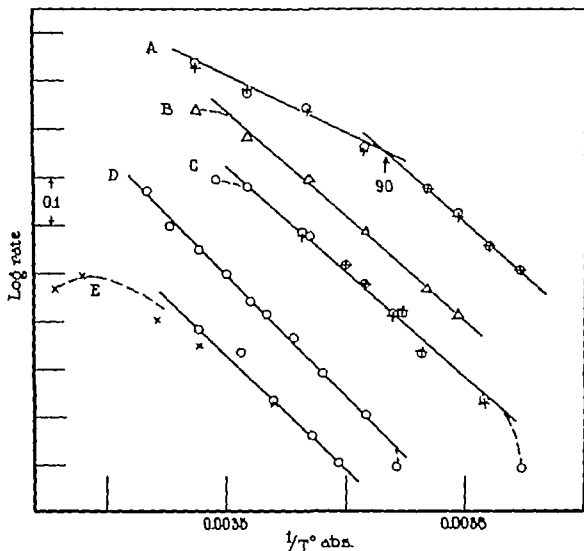


FIG. 8 Data upon the development of teleosts

A *Gadus morhua* and *G. aeglefinus*, B, *G. merlangus* average velocities of development up to hatching: observations by Dannevig (in Johansen and Krogh, 1914) for A $\mu = 11,800$ and $\mu = 20,200$ with break at 9.0°C for B $\mu = 20,000$

C Plaice to length 4.6 mm. circles to length 4.9 mm. crosses the two series brought together by multiplying the members of the second by a factor $\mu = 20,000$ (Johansen and Krogh, 1914)

D, *Hypomerus olidus* (Higurashi and Tauti 1925) $\mu = 23,700$

E *Plecoglossus altivelis* two series one from Higurashi and Tauti (1925) the second from Higurashi and Nakai (1926) $\mu = 23,000$

It should be noted that in the cases of deviation at extreme temperatures there is independent evidence of abnormal differentiation.

development of the frog, in Krogh's experiments, exhibits a constant increment not significantly different from that for the mid-range of temperatures (15° to 47°) in the case of the first cleavage, but the critical temperatures are different

It is noteworthy that in the case of amphibian development the temperature characteristics calculated from the data of Lillie and Knowlton (1897) upon the early development of *Rana* and of *Amblystoma* (Fig 7) may be said to agree quantitatively with the magnitude obtained over the lower temperature range (4° to 15°) in Krogh's (1914) experiments

The rate of development of teleost embryos has been studied by Krogh and others. The more extensive series of observations are collected in Fig 8. More recent experiments on the rate of insect development provide data showing for *Dytiscus marginalis* $\mu = 19,300$, for *D. semisulcatus* $\mu = 20,000$ (to 15°) and $\mu = 10,400$ (data from Blunk, 1923). Some figures from Ziegelmayer (1926) for rate of development of *Cyclops* give $\mu = 15,700$. Such data have been treated in a different way by Krogh (Johansen and Krogh, 1914), but the most direct utilization of the measurements shows that there is a striking constancy in the occurrence of $\mu = 20,000$, with one series showing $\mu = 24,000$, thus there is evidenced a suggestive parallelism with the values obtained for amphibian development.

For the full interpretation of such graphs it is necessary to know the effect of the preliminary period during which the objects (*e g*, fertilized eggs) have been maintained at some constant or nearly constant temperature before distribution to thermostats at different temperatures. In the light of the view previously set forth in this paper it would be expected that if at all prolonged this preliminary interval might well have a definite and detectable effect. It would be expected to accentuate any innate departure from the rectilinear character of the plots, or might seriously affect the apparent magnitude of the temperature characteristic. This especially makes it impossible to use critically much of the early material on insect development (*cf* Sanderson, 1910, Sanderson and Pears, 1913), although it supplies interesting suggestions. In the most carefully conducted experiments the

adherence to rectilinearity is certainly satisfactory (*cf* Crozier, 1924-25 b, Bliss, 1925-26, Brown, 1926-27)

The fact that the same temperature characteristic holds for mean growth velocities at different points on a curve of development (Fig 7) must be taken to signify that within the range considered the shape of the underlying curve very nearly is the same at different temperatures, hence, that only one "velocity constant" is materially effective or else that if more than one be involved, their temperature characteristics are the same⁴

The net result may be stated by saying that while the control of growth velocities by chemical reaction velocities seems adequately shown, it is yet highly desirable that further data be secured by improved methods, there is some indication that the controlling reactions may belong in categories with those found by their temperature characteristics to be implicated in other and quite diverse vital processes. There is as yet scarcely sufficient evidence to verify the prediction that the curve relating log velocity of growth to $1/T^{\circ}$, when "velocity" = reciprocal of time required to reach a defined stage, should be slightly curvilinear. But there is indication that growth velocities, where evidenced as constant rates of increase, adhere satisfactorily to the Arrhenius formula, and even when we may quite reasonably expect that an "autocatalytic" system is involved, the agreement is often as good as might be desired. The values of the temperature characteristics secured for growth phenomena are quite varied, yet they cluster rather definitely about the following magnitudes 7-8,000, 11-12,000, 16-17,000, 20,000, 24,000, 27,000 (this summary is based upon studies of a number of cases additional to those specifically mentioned in this article). Consideration of the properties of the equation which describes the velocity of an autocatalytic process (Section IV) shows how this sort of result may be obtainable.

⁴ With regard to velocities of regenerative growth which in certain cases at least appear to adhere to typical growth curves there does not exist any considerable body of data. We may cite the following instances. The regeneration of hydranth in *Tubularia* (Moore 1910) has been cited in an earlier paper (Crozier, 1924-25 b). Measurements of the rate of regeneration (morphyllaxis) in planarians indicate a high temperature coefficient (Lillie and Knowlton 1897, Vandel 1921-22) but are insufficient for analysis.

VI

SUMMARY

The velocity of growth, taken as the reciprocal of the time required to attain a given size or stage of development, obeys with some exactness the Arrhenius equation for relation to temperature. The values of μ , and the type of "breaks" found in the curves connecting velocity and temperature, are similar to those found in the case of various other vital activities. More precise data, particularly from experiments in which parts of the given developmental stadium are passed at different temperatures, may strengthen present indications that this relationship is not absolute. It is pointed out that the equation for an autocatalytic process, taken as descriptive for growth, predicts particular sorts of deviation under these conditions, which have in one instance been obtained experimentally, and may at the same time nevertheless permit the apparent temperature characteristic for (average) growth velocity to agree rather closely with that for one of the two velocity constants present in the correct autocatalytic equation.

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EXIT OF DYE FROM LIVING CELLS OF NITELLA AT DIFFERENT pH VALUES *

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I

INTRODUCTION

The purpose of the present paper is to outline a theory¹ of the penetration of a dye (brilliant cresyl blue) into living cells of *Nitella*,² and to examine how far this theory is in harmony with the facts found in studying the exit of the dye from the cell

* This work was in part done when the writer held a Fellowship in the Biological Sciences of the National Research Council, Washington D C.

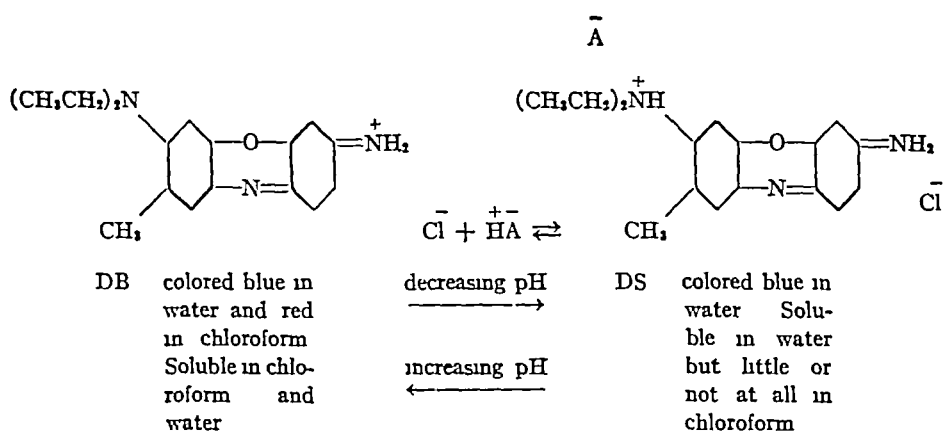
¹ Another theory previously proposed by the writer (Irwin M. *J Gen Physiol* 1922-23 v 727) regards the rate of penetration and the final equilibrium as dependent primarily on the concentration of the salts of proteins or weak acids, NA at the surface of the cells which combine with DS to form a compound capable of diffusing into the sap But on further experimentation the writer has concluded that there are many objections to this theory The most serious objection of all is found in the fact that the rate of penetration seems to be directly proportional to the ratio $\frac{\text{DB}}{\text{DS}}$ for each particular dye at various pH

values, when a comparison of the relative rates at these pH values is made by the writer among several basic dyes having different apparent dissociation constants.

Penetration is regarded by the following writers as dependent on the combining of dye ions with proteins Bethe, *A. Biochem. Z.* 1922 cxxvii, 18. Rohde K. *Arch ges Physiol* 1920 cxxxii 114 Pohle E. *Deutsch med Woch* 1921 xlvii 1464 Collander R. *Jahrb wissenschaft Bot* 1921 lx, 354 Mathews, A. *Am J Physiol* 1898, i, 445

² *Nitella* is a fresh water plant with multinucleate cells up to 4 inches in length, having an outer cell wall, beneath which is a very thin laver of protoplasm surrounding a relatively large central vacuole. The pH value of the sap in the vacuole is about 5.6 and the sap contains about 0.1 M halides in addition to organic acids and protein

The theory^{3,4} states that the dye exists in (at least) two forms, one of which is the "free base"⁵ which we may call DB, soluble in chloroform, and another, which we may call DS, little or not at all soluble in chloroform. When the pH value of the solution increases a part of DS is changed to DB (and *vice versa*), so that at each pH value these two forms are in equilibrium with each other (and possibly with a third form, which may be a pseudo base found at still higher pH values). The nature of DB is still an open question. According to the theory of Hantzsch and others⁶ both DB and DS may be treated as salts, DB being a quaternary ammonium salt which is capable of undergoing a further salt formation owing to the presence of other basic groups. The following⁷ may make this clear by using cresyl blue as an example.



³ Irwin, M, *J Gen Physiol*, 1925-26, viii, 147

⁴ Irwin, M, *J Gen Physiol*, 1925-26, ix, 561

⁵ In former papers (see Foot-notes 3 and 4) this free base was called DOH for convenience but in order to avoid any possibility of confusing DOH with the dye hydrate (which may not be the form we are dealing with), it will be called DB hereafter

⁶ For a discussion of the theory of indicators see Henrich, F, *Theories of organic chemistry*, translated by John Johnston and Dorothy Hahn, London, 1922

⁷ For the formula see Conn, H. G., *Biological stains*, Geneva, New York, 1925, 51

In the case of brilliant cresyl blue, DB and DS have the same color. If the above description of DB and DS is correct DB may be a strongly dissociated salt like DS. On the other hand, DB may be regarded as an undissociated molecule, and DS a strongly dissociated salt. Experiments are being carried out by the writer to determine the behavior of DB in this respect. Dr Grinnell Jones has kindly determined the change in the conductivity of chloroform with and without the dye. When 100 cc. of pure chloroform were shaken up with 1 liter of $M/150$ borate buffer solution at pH 9, the specific conductivity of this chloroform was found to be 6×10^{-10} . When the same volume of chloroform was shaken up with 1 liter of $M/150$ borate buffer solution at pH 9 containing $3.5 \times 10^{-4} M$ brilliant cresyl blue until there was practically no dye left in the aqueous solution, the specific conductivity of this chloroform was found to be 233×10^{-10} (about forty times greater than that of the chloroform containing no dye). This indicates that some or all of the dye exists in the chloroform in dissociated form.

The behavior of these two forms is very different. Apparently⁸ DB can pass through the cell rapidly but DS penetrates extremely slowly or not at all.

Although it is evident that the form of the dye⁹ which principally

⁸ In connection with this it may be assumed that DS corresponds with the ions and DB with the undissociated molecules of a weak base, acid, or salt. In the paper by Hoagland and Davis (Hoagland D. R. and Davis A. R. *J Gen Physiol* 1923-24 vi 47) it is stated that the time of exposure of the living cells of *Nitella* to solutions containing NO_3 or Br ions is a matter of days before a detectable amount is found in the sap even at a favorable external pH value, temperature and condition of light. The time of exposure on the other hand in the case of the penetration of cresyl blue into *Nitella* at a favorable external pH value, and temperature is a matter of seconds. This fact agrees very well with Osterhout's suggestion (see Foot note 10) that the undissociated molecules enter the cell, while the ions enter only very slowly or not at all. Furthermore it agrees with the writer's theory since the halides are only very slightly soluble in substances like chloroform and benzene and in this respect the halides correspond with DS of the basic dye.

⁹ The following writers state that some basic dyes enter the living cells as a free base. Overton E. *Jahrb wissenschaft Bot* 1900 xliii 669. Harvey E. N. *J Exp Zool* 1911 x, 507. Robertson T. B. *J Biol Chem*. 1908 iv 1. McCutcheon M. and Lucke B. *J Gen Physiol* 1923-24 vi 501. It is stated by Brooks, M. M., *Am. J Physiol* 1926 lxxvi 360 that an acid dye 2,6-dibromophenol indophenol penetrates *Valonia* only in the form of an undissociated molecule.

penetrates the cell is the one which is soluble in chloroform, the writer does not wish to subscribe without reserve to the lipoid theory in its present form

Previous experiments^{1,2} have shown that when the external concentration is kept constant throughout the experiment, the entrance of the dye is found to follow the equation

$$\frac{dx}{dt} = k(a - x)$$

where a = the concentration of the dye in the sap at equilibrium and x = the concentration of the dye in the sap at the time t , while k = the velocity constant. When the values of x are calculated from this equation they are found to agree very closely with the observed

This agreement of course does not determine whether the process is governed by diffusion or by chemical reaction

The temperature coefficient, furthermore, for the rate of penetration between 20°C and 25°C is very high (above 4) but this again may not necessarily indicate that the process is controlled by a chemical reaction rather than by diffusion

Until further knowledge is obtained concerning the temperature coefficient for the diffusion of substances through an artificial system which more or less closely resembles the living cell of *Nitella*, and in which the passage of solute molecules or ions from one solvent phase to another probably does not depend upon forces of the sort usually regarded as "physical" it is not possible to determine whether the rate is governed by simple diffusion or by chemical reaction

It is quite possible that under some circumstances it is controlled by diffusion and under other circumstances by chemical combination

Since we are unable at present to decide whether the rate is controlled by diffusion or by chemical reaction let us for the sake of simplicity assume that it is diffusion, since in this case the mechanism is less complicated, and proceed to analyze the data on this basis. After this is done we shall discuss the alternative hypothesis, *i e.*, that the rate is controlled by chemical reaction

If we assume that the rate is controlled by diffusion, the mechanism may be explained as indicated by Diagram A (the cell wall being omitted). In this diagram nothing is said regarding combination of

the dye with a cell constituent but this does not indicate that there is no possibility of such a reaction in the protoplasm. It is regarded, for the present, as not affecting the rate, it is therefore omitted in order to simplify the diagram

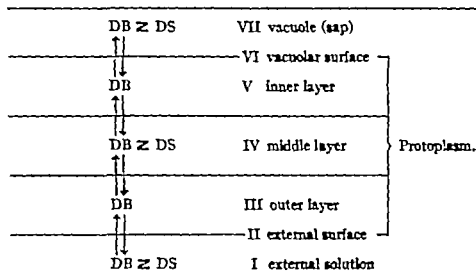


Diagram A. The cell wall is omitted for convenience.

Diffusion is designated by the sign \rightleftharpoons , equilibrium between DB and DS by the sign \rightleftharpoons . For convenience the process is divided into seven parts. The inner and outer layers (III and V) are hypothetical. The vacuolar surface (VI) represents the protoplasmic surface in immediate contact with the sap, while the external surface (II) represents the protoplasmic surface in contact with the external solution. In I, IV, and VII, DB is in equilibrium with DS and a constant ratio of $\frac{DB}{DS}$ is maintained in each medium as long as the conditions remain unchanged. The concentration of DB in one part is in definite relation with that of DB in any other part of the diagram. Thus, for example, if the concentration of DB in the external solution (I) is changed, successive changes in the concentration of DB in all the parts of the cell take place. It is assumed that DB diffuses through III and V while DS diffuses to such a slight extent as to be negligible in the present case. For penetration the velocity of diffusion of the dye, DB, from I to VII is greater than that from VII to I while for the exit the velocity from VII to I is greater. An equilibrium is established when the velocity of the inward diffusion is equal to the outward

diffusion At equilibrium the concentration of DB in the vacuole is proportional to the concentration of DB in the external solution depending on the apparent¹⁰ dissociation constant and on the distribution coefficient $C = \frac{\text{DB in the sap}}{\text{DB in the external solution}}$ If C is 1, the concentration of DB in the sap at equilibrium is equal to the concentration of DB in the external solution If C is lower than 1, the concentration of DB in the sap will be lower than that of DB in the external solution, and *vice versa* Since there is maintained in the sap a definite ratio of $\frac{\text{DB}}{\text{DS}}$, the concentration of DS depends on the concentration of DB Thus the final concentration of the total dye (DB and DS) in the sap at equilibrium will depend on the apparent dissociation constant of the dye (i.e. the ratio of $\frac{\text{DB}}{\text{DS}}$) in the sap, on the partition coefficient of DB, and on the ratio of $\frac{\text{DB}}{\text{DS}}$ in the external solution

According to this scheme it is possible to study the mechanism either of the penetration of the dye into or of the exit from the vacuole, by determining the concentrations of the dye (DB plus DS) in the sap, as long as the color of DB does not differ from that of DS The rate of penetration will increase and that of exit will decrease when the concentration of DB just outside the external surface (II) is increased The reverse is the case when the concentration of DB in the sap is increased as, for example, by any change in the medium which changes the apparent dissociation constant of the dye (i.e. the ratio of $\frac{\text{DB}}{\text{DS}}$), or by a change in the solubility of DB in the sap

The theory thus outlined accords with the facts previously obtained for the penetration of dye^{3 4} Let us now consider whether it accords with the facts observed in connection with the exit of dye from the cell We shall proceed upon the assumption that when a stained cell is placed in a solution containing no dye, the dye comes out according to

¹⁰ Osterhout, W J V, *J Gen Physiol*, 1925-26, viii, 131 Osterhout, W J V, and Dorcas, M J, *J Gen Physiol*, 1925-26 ix, 255

the scheme outlined in Diagram A (the outward process VII I from the sap to the external solution) We shall test this assumption by experiments

II

Methods

Living cells of *Nitella* were placed in $8.6 \times 10^{-4} M$ brilliant cresyl blue at pH 8.2 for 17 minutes, when the concentration of the dye in the sap reached $7.94 \times 10^{-4} M$ (The concentration of dye in the sap was determined colorimetrically as described below) The cells were then removed from the dye solution, gently wiped with a damp cloth, and distributed in solutions at different pH values (pH 5.4 to 8.2) containing no dye At definite intervals a few cells were removed, and the concentration of the dye in the sap was determined by the colorimetric method as follows The end of each cell was cut and the sap was gently squeezed out onto a glass slide, the sap was drawn up into a capillary tube the color of which was matched with that of the capillary tube of the same diameter containing a standard dye solution

In order to avoid experimental error from the presence of the dye in the external solution, only six cells were placed in 200 cc. of solution (without dye) and the solution was constantly stirred and changed every 15 seconds This method gives the maximum velocity constant for each experiment, i.e. there is no further increase in the velocity of the exit of the dye if the frequency of stirring and of changing the solutions is increased

The concentration of the external dye solution ($8.6 \times 10^{-4} M$) is chosen because this is sufficiently dilute to avoid error due to the adhering of the dye to the surface of the cell, after the cell is removed and wiped with a damp cloth The cellulose wall is not stained when cells are placed in this concentration of dye at pH 8.2

If too high an external dye concentration is used, the exit of the dye from the sap is hindered (even when the cell wall is not stained) when the cells are removed from the dye solution and placed in a solution without dye, though the latter solution is constantly stirred and changed This decrease in the rate of the exit of the dye is due in all probability to the fact that the dye adhering to the surface of the cell cannot be washed away quickly enough This complication may

be eliminated by using an external solution which is at least seven times more dilute than the concentration of the dye in the sap which is chosen for the experiments. In order to be absolutely certain that the concentration used avoids this experimental error the experiment was repeated with still lower concentrations but it was found that the result was not altered.

All possible care was taken to have all the cells used at one time as alike as possible, so that the differences in the rates were due chiefly to the experimental conditions and not due to the difference in the condition of the cells before the experiments began. Unless otherwise stated, the *Nitella* used was obtained from Cambridge and the experiments were carried out in early fall when the cells were in excellent condition.

The tests for early stages of injury are very unsatisfactory. The appearance of masses of chlorophyll in the expressed sap, the rapid exit of halides from the intact cell, and the loss of turgidity all indicate advanced stages of injury rather than the first. For this reason it is desirable to control the experiments in some way so that we have a more or less uniform method of detecting the condition of the cell immediately after the experiments. To do this, after each experiment, some of the cells were tested for injury by placing them in distilled water, and for 4 days¹¹ at intervals of every few hours the percentage mortality was compared with that of the control cells (fresh cells placed in distilled water under same conditions). It was found that the percentage mortality of the cells thus treated was about the same as that of the control cells.

These experiments, like those heretofore described^{3,4} by the writer, were carried out in an incubator at $25 \pm 0.5^{\circ}\text{C}$, into which diffused light was permitted to enter through small ventilating holes.

The buffer solutions used were M/150 phosphate mixtures. The pH values of these buffer solutions were determined by means of the hydrogen electrode. The dye used was that of Grubler, and was taken from the same stock bottle as the one used in the writer's experiments^{3,4} on penetration.

¹¹ It is not desirable to continue such a test for any longer period since the comparison between the test cells and the control cells becomes more doubtful, in view of the fact that even the control cells do not live indefinitely in the laboratory.

III.

Analysis of the Time Curves

That lowering of the pH value of the external solution (containing no dye) hastens the exit of the dye from the sap of living cells of *Nitella* is indicated¹² by the curve in Fig 1. At low pH values (5.4 to 6) the process may be followed until practically all the dye has come out of the sap without causing injury to the cells, but at higher pH values injury or death may occur. The curves given in Fig 1 represent the process when the cells are not injured.

At higher pH values of the external solutions here employed it is probable that all the dye in the sap would eventually be found to come out of the vacuole if we could continue the experiment long enough and still keep the cell from being injured. The analysis of the time curves therefore is made on the assumption that at the end of the process the concentration of the dye in the sap is zero at all external pH values.

The velocity of diffusion is assumed to be proportional to the difference between the concentration of DB in the sap and that of DB in the external solution. According to the present theory, there is a definite ratio of $\frac{DB}{DS}$ in the sap and in the external solution, so that for mathematical treatment the concentration of DB in both may be replaced, for convenience, by the concentration of the total dye (DB and DS) which we actually measure. Since the concentration of the dye in the external solution is approximately zero, we may in the following equation let a denote the initial concentration of the dye in the sap, x the concentration of the dye that has disappeared from the sap at time t , and k the velocity constant of diffusion. We may then write

$$\frac{dx}{dt} = k(a - x) \quad \text{or} \quad k = \frac{1}{t} \log \frac{a}{a - x}$$

When k is calculated for each time curve it is found to decrease

¹² These results confirm those obtained previously by the writer (Irwin M. *J. Gen. Physiol.*, 1922-23 v 223). It may be added here that the writer has chosen to study the exit of the dye by the method presented in this paper first, because other methods offer greater complications.

TABLE I
Exit of Brilliant Cresyl Blue from Living Cells of *Nitella* at Varying External pH Values at 25°C

The process is represented by the equation $\frac{dx}{dt} = k(a - x)$, where a is the initial concentration of dye in the sap, $a - x$ is the concentration of dye in the sap at the time t , and k is the velocity constant. The calculation is made with a 20 inch slide rule $a = 7.94 \times 10^{-5} M$ for all external pH values

t	pH 5.4			pH 5.7			pH 6.0			pH 6.8			pH 7.5			pH 7.8			pH 8.2		
	$a - x$ obs.	k	$\frac{a - x}{\text{calc.}}$	$a - x$ obs.	k	$\frac{a - x}{\text{calc.}}$	$a - x$ obs.	k	$\frac{a - x}{\text{calc.}}$	$a - x$ obs.	k	$\frac{a - x}{\text{calc.}}$	$a - x$ obs.	k	$\frac{a - x}{\text{calc.}}$	$a - x$ obs.	k	$\frac{a - x}{\text{calc.}}$	$a - x$ obs.	k	$\frac{a - x}{\text{calc.}}$
min	$M \times 10^5$		$M \times 10^5$	$M \times 10^5$		$M \times 10^5$	$M \times 10^5$		$M \times 10^5$	$M \times 10^5$		$M \times 10^5$	$M \times 10^5$		$M \times 10^5$	$M \times 10^5$		$M \times 10^5$	$M \times 10^5$		$M \times 10^5$
1	4.83	0.215	4.96	4.83	0.215	5.07	5.59	0.152	5.62	6.21	0.107	6.30	6.14	0.055	6.02	6.56	0.041	6.46	6.9	0.030	7.08
2	3.05	0.208	3.11	3.28	0.192	3.23	4.00	0.148	3.96	4.83	0.107	5.01	5.00	0.067	5.24	5.87	0.043	5.82	6.80	0.023	6.69
3	2.15	0.189	1.94	2.31	0.179	2.06	2.76	0.153	2.81	4.14	0.094	3.98	3.80	0.064	3.97						
5							1.40	0.151	1.40	2.76	0.092	2.51	3.28	0.055	3.02	3.80	0.046	3.84	5.52	0.023	5.31
7																2.59	0.049	2.82	4.49	0.025	4.47
10																					
Average	0.204			0.195			0.151			0.100			0.060				0.045			0.025	

slightly as the concentration of the dye (DB and DS) in the sap approaches zero (Table I). This decrease in the value of k may be explained on the ground that there is a relative increase in the velocity of the inward process due to an increase in the ratio of DB in the film just outside the external surface (II) to the DB in the outer layer (III) and hence to the DB or to the total dye (DB and DS) in

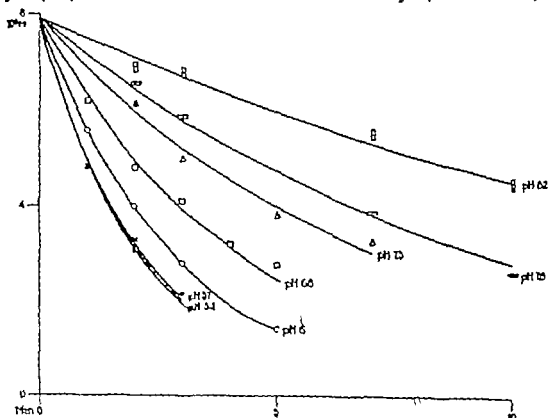


FIG. 1. Time curves showing the extent of brilliant cresyl blue from the living cells of *Nitella* at different external pH values at 25°C., when the initial concentration of the dye in the sap is 7.94×10^{-4} M. The ordinates represent the concentrations of dye in the sap while the abscissae represent time. The curves as drawn represent the calculated values of the concentration of the dye in the sap while the symbols represent the observed values. Each point on every curve is an average of fifty experiments, and the probable error of the mean is less than 8 per cent of the mean.

the sap, since toward the end of the process, where there is a very little dye (DB and DS) left in the sap, the amount of DB in the film just outside the external surface (II) may no longer be a constant fraction of DB in the outer layer (III) and of the total dye in the sap as was the case at the beginning of the process (this will be discussed later on). Since this decrease in the con-

stants is not very great, the average is taken of all the velocity constants at each external pH value. When the values of $a - x$ are calculated for each time curve by using the average value of k , thus obtained, they are found to agree fairly closely with the observed except toward the end of the process, where there is an indication that the calculated values are slightly lower than the observed, as shown under pH 5.4 and 5.7, Table I.

In connection with the analysis of the time curves it may be well to repeat the following in order to avoid misunderstanding. (1) It makes no difference in the form of the time curve whether we measure DB alone or DB + DS in the sap, since DB and DS stand in constant relation as long as the conditions, such as the pH value of the sap, remain unchanged. We actually measure DB plus DS (called the "total dye" for convenience) in the sap and the analysis of the time curves is made by using the concentrations of the total dye. (2) The concentration of the total dye in the sap is affected by the concentration of DB in the other parts of the cell, and in the solution outside the cell. Thus, for example, if the concentration of DB in the outer layer (III in Diagram A) is decreased, the concentration of DB and hence that of the total dye in the sap is decreased.

When the temperature coefficient between 20° and 25°C, for the exit of the dye at pH 5.7 and also at pH 7.8 was determined, Q_{10} was found to be about 4.

IV

The Relation of the Velocity Constant to the pH Value of the External Solution

The time curve for each external pH value is found (see Table I) to follow approximately the equation

$$\frac{dx}{dt} = k(a - x)$$

where a denotes the initial concentration of DB in the vacuole minus the concentration of DB in the external solution (which in this case is practically zero), x the amount of DB that has diffused out of the vacuole at the time t , and k the velocity constant. In both cases, DB for convenience is put equal to the total dye which is actually measured.

If the stirring and frequent changing of the external solution kept the concentration of DB equal to zero just outside the external surface of the protoplasm (which is designated by II, the external surface, in Diagram A), we should expect to find the same values of k for all external pH values. But the value of k decreases with an increase in the external pH value, as shown in Fig 2, and the explanation for

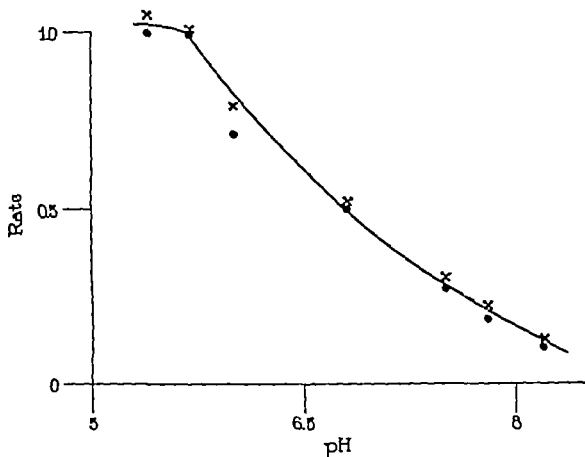


FIG 2 Curve showing the relation of the external pH values to the rates of the exit of the dye (symbol X) and also to the velocity constants (symbol ●). The ordinates represent the rates, and also the velocity constants multiplied by 5.2 (for convenience of plotting) and the abscissae represent the pH values of the external solutions.

this may be that the concentration of DB at the external surface changes as the external pH value changes. Let us assume that just outside the external surface of the protoplasm there is a liquid film more or less protected against the direct effect of stirring by the cellulose wall and that in this film a certain amount of DB (a certain percentage of which is at once transformed to DS) collects as it comes out of the cell, also that the total amount of DB

which comes out is approximately the same in all solutions containing no dye, but the per cent of it which remains in the form of DB depends on the pH value of this film (which is assumed to be approximately the same as that of the external solution since the latter can penetrate freely through the cellulose wall into the film) since a certain amount of DB will change to DS depending on the pH value of the film

When the cell is removed from the dye, wiped, and placed in a solution in which no dye is present, DB begins to diffuse from the vacuole, the protoplasm, and the film just outside the protoplasmic surface. We may assume that the concentrations in all of these places fall off together, so that when the concentration in the vacuole has fallen to half the value it had at the start, that of the protoplasm and the film will also have fallen to approximately half value. In that case we may regard the falling off in the protoplasm and in the film as following an approximately unimolecular curve (since we have found this to be true of the dye in the vacuole) and consequently the amount of DB in the film will be an approximately constant fraction of that in the sap. If we call the dye in the sap $a - x$ and designate as y the amount of DB in the very thin layer in immediate contact with the outer surface of the protoplasm we may write

$$y = b (a - x)$$

in which b is a constant expressing the amount of DB in the film as a fraction of the amount of DB (which for convenience is put equal to the total dye) in the vacuole throughout the process at any one external pH value

When the pH value of the external solution changes the value of b will also change, since the per cent of DB in the film will be altered. In order to see how this will affect the rate of exit of the dye from the vacuole, let us first consider the case where there is no effect of y on the velocity constant. Since according to our analysis of the time curves, the dye comes out of the vacuole in a unimolecular fashion, we may write

$$\frac{dx}{dt} = k_1 (a - x)$$

in which k_1 is the velocity constant of the process when dye is present

on one side of the surface only This expression gives us the rate of exit of the dye when there is no dye in the film. When dye is present in the film a certain amount diffuses back into the cell The true rate¹²

¹² Criticism may be made as to this method of mathematical treatment since it involves the consideration of the diffusion of DB through only one very thin surface when in fact the protoplasm of *Nitella* consists of more than one such layer Even if we were to treat the entire protoplasmic layer as one surface, the question may be raised as to how far we are justified in considering the protoplasm to be thin enough for such a mathematical treatment. If we consider the diffusion of DB through two surfaces the vacuolar and the external surfaces (II and VI in Diagram A) one at a time then we may modify the analysis given in the text in the following manner The amount diffusing inward through the external surface in unit time when DB is present in the film only = k_1y (just as described in the text) Let us assume that the amount diffusing inward in unit time through the vacuolar surface when there is no DB in the vacuole is a constant fraction of k_1y so that we may put this amount equal to ck_1y in which c is a constant. The amount diffusing outward in unit time through the vacuolar surface when DB is present in the vacuole but not in the protoplasm or in the film (DB fictitiously introduced into the vacuole without getting into the protoplasm) is $k_1(a-x)$ Hence we take the difference between the amount going outward through the vacuolar surface and the amount passing inward through the vacuolar surface and we have

$$\frac{dx}{dt} = k_1(a-x) - k_1cy$$

put $y = b(a-x)$ in which b is a constant (just as given in the text) then

$$\frac{dx}{dt} = k_1(a-x) - k_1bc(a-x)$$

$$\frac{dx}{dt} = (k_1 - k_1bc)(a-x)$$

or on integration

$$k_1 - k_1bc = \frac{1}{t} \log \frac{a}{a-x}$$

$$bc = \left(k_1 - \frac{1}{t} \log \frac{a}{a-x} \right) + k_1$$

Since we are not able to verify the values of the constants b and c experimentally, and since assuming a value for either b or c is very unsatisfactory we are not able to explain the mechanism any more convincingly than we have done in the text.

Experiments are now in progress to see whether it is possible to determine the

of exit is the resultant of these two processes and may be found by subtracting the amount which would diffuse inward if dye were present on one side only from the amount that would diffuse outward if dye were present on the other side only. Hence we may write

$$\frac{dx}{dt} = k_1 (a - x) - k_1 y$$

Substituting in this equation the value $y = b (a - x)$ we have

$$\frac{dx}{dt} = k_1 (a - x) - k_1 b (a - x)$$

$$\frac{dx}{dt} = (k_1 - k_1 b) (a - x)$$

or, on integration,

$$k_1 - k_1 b = \frac{1}{t} \log \frac{a}{a - x}$$

$$k_1 (1 - b) = \frac{1}{t} \log \frac{a}{a - x}$$

$$k_1 = \left(\frac{1}{t} \log \frac{a}{a - x} \right) \div (1 - b)$$

and

$$b = \left(k_1 - \frac{1}{t} \log \frac{a}{a - x} \right) \div k_1$$

We may put $k_1 (1 - b) = k$, substituting the value $k = \frac{1}{t} \log \frac{a}{a - x}$ we have $b = \frac{k_1 - k}{k_1}$

constants experimentally in order that we may know in greater detail what the controlling factor is for the rate of penetration into and that of exit of the dye from the vacuole

It might be possible that the rate of penetration into and that of the exit of the dye from the vacuole are controlled by the rate of diffusion of DB through only one very thin layer in the cell, (the layer through which the diffusion of DB is the slowest). Whether this is represented by the external surface (II in Diagram A) or by the vacuolar surface (VI) or by some other part of the cell, we are not able to state definitely at present. In all probability under varying conditions the controlling layer varies

The values of a , x , and t may be obtained experimentally but the values of k_1 and b cannot be obtained in this way. We may, however, assume an approximate value of k_1 and we are justified in doing this since we are interested in relative rather than in absolute values. The analysis of the time curves shows decreasing values of k (see Table I and Section III) with increasing external pH values. When such

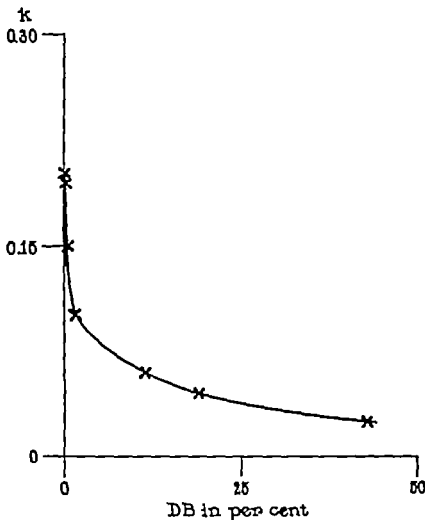


FIG. 3 Curve showing the relation of the velocity constants (k) plotted as ordinates to the concentrations of free base (DB) in per cent as abscissæ.

values of k are plotted as ordinates and the concentration of DB in the external solution expressed as per cent of the total dye, (obtained from the experiments made by the writer on the distribution of the dye between chloroform and water) are plotted as abscissæ, we obtain the curve shown in Fig. 3. The curve indicates that when DB (and consequently y) equals zero, the value of k is somewhat above

TABLE II

The Relation of the Dye in the Film Just Outside the Protoplasmic Surface to the Dye in the Vacuole, at Different External pH Values

The values of b are obtained by the equation $b = (k_1 - \frac{1}{t} \log \frac{a}{a-x}) - k_1$ where k_1 denotes the velocity constant of the diffusion of the dye into or out of the living cell of *Nitella* where dye is present on one side of the protoplasmic surface only (the value of k_1 is assumed to be 0.37), where a denotes the initial concentration of dye in the vacuole, x the amount of dye that has diffused out of the vacuole at time t , and where b is a constant expressing the concentration of DB in the film as a fraction of the concentration of DB in the vacuole throughout the process at any one external pH value. Knowing the values of b , the values of y are obtained by the equation $y = b(a-x)$ where y denotes the DB in the film, $a-x$ the DB in the vacuole. Values of y thus obtained are relative values, since the given observed values of $a-x$ represent the "total dye" (DB + DS). Calculation is made with a 20 inch slide rule

pH 5.4				pH 5.7				pH 6.0				pH 6.8			
$a-x$ obs.	k	b	y when $b = 0.45$	$a-x$ obs.	k	b	y when $b = 0.47$	$a-x$ obs.	k	b	y when $b = 0.59$	$a-x$ obs.	k	b	y when $b = 0.73$
$M \times 10^3$			$M \times 10^3$	$M \times 10^3$			$M \times 10^3$	$M \times 10^3$			$M \times 10^3$	$M \times 10^3$			$M \times 10^3$
4.83	0.215	0.42	2.17	4.83	0.215	0.42	2.27	5.59	0.152	0.59	3.30	6.21	0.107	0.71	4.55
3.05	0.208	0.44	1.37	3.28	0.192	0.48	1.54	4.00	0.148	0.60	2.36	4.83	0.107	0.71	3.53
2.15	0.189	0.49	0.97	2.31	0.179	0.52	1.09	2.76	0.153	0.59	1.63	4.14	0.094	0.75	3.02
								1.40	0.151	0.59	0.83	2.76	0.092	0.75	2.02
Average		0.45				0.47				0.59				0.73	

pH 7.5				pH 7.8				pH 8.2			
$a-x$ obs.	k	b	y when $b = 0.84$	$a-x$ obs.	k	b	y when $b = 0.88$	$a-x$ obs.	k	b	y when $b = 0.93$
$M \times 10^3$			$M \times 10^3$	$M \times 10^3$			$M \times 10^3$	$M \times 10^3$			$M \times 10^3$
6.14	0.055	0.85	5.16	6.56	0.041	0.89	5.78	6.90	0.030	0.92	6.42
5.00	0.067	0.82	4.20	5.87	0.043	0.89	5.16	6.80	0.023	0.94	6.32
3.80	0.064	0.83	3.19	3.80	0.046	0.88	3.35	5.52	0.023	0.94	5.14
3.28	0.055	0.85	2.76	2.59	0.049	0.87	2.27	4.49	0.025	0.93	4.18
Average		0.84				0.88				0.93	

0.3 Extrapolation has been attempted by various methods but with such a curve it is very difficult to obtain any reliable result We may assume, however, that we are not too far from the true value if we take the maximum value of k to be 0.37

If we solve for the values of b in the above equation, we find that they remain fairly constant for each external pH value, but they increase with an increase in the external pH value as shown in Table II It may be stated here that the values of b are the same whether $a - x$ represents the "total dye" or DB

Knowing the values of b and $a - x$, we may calculate the values of y by means of the equation $y = b(a - x)$ for any value of $a - x$ as shown in Table II

In calculating the values of y , the observed values of $a - x$ (Table II) representing the "total dye" in the sap are used for convenience Since we are interested primarily in the relative values of y , such values will give us the desired information It is needless to state that if the values of DB in the sap were used instead of those of the total dye (DB plus DS), the values of y would be considerably lower than those given in Table II, but the ratio of one value of y to another would remain unchanged

At each external pH value the values of y are found to increase with increase in the value of $a - x$ If we take a fixed value of $a - x$ and compare the values of y at different pH values, we find that the value of y increases with an increase in the external pH value

In order to bring out clearly the effect of y on the velocity constant of exit we may return to the equation on page 90

$$k = k_1 - k_1 b$$

and substitute the value $b = \frac{y}{a - x}$ (see page 88) We then have

$$k = k_1 - \frac{k_1 y}{a - x}$$

It may be added here that the values of k are the same whether $a - x$ represents the "total dye" or DB

Let us now consider the relation of y to the per cent¹⁴ of DB in

¹⁴ The discussion of the apparent dissociation constant is given in detail in the paper referred to in Foot note 3

the film (as determined by the pH of the external solution) We shall take for convenience the values of y where $a - x$ is 4.5×10^{-5} at different external pH values and take the percentage of DB as calculated from the distribution of DB between chloroform and water at different pH values of the external solution

Let us first see if the values of y at different pH values are proportional to the values in per cent of DB obtained from the dissociation³ curve of the dye (which gives the DB in per cent calculated from the data obtained by the experiments on the distribution of the dye between chloroform and water) If we take for convenience the value 4.5×10^{-5} M for $a - x$ and find the value of y at pH 7.8 at which pH value 20 per cent of the dye is in the form of DB (according to the dissociation curve) we are able to calculate the values of DB in per cent on the basis of the values of y at other pH values (since we know the values of y for this fixed value of $a - x$), by the following equation

$$\frac{y_1}{y_2} = \frac{m_1}{m_2}$$

when y_1 = the value of y at pH 7.8 = 4×10^{-5} M

y_2 = the value of y at another pH value, say pH 7.5 = 3.8×10^{-5} M

m_1 = 20 per cent

m_2 = the DB in per cent at pH 7.5

By substituting we get

$$\frac{4 \times 10^{-5}}{3.8 \times 10^{-5}} = \frac{20}{m_2}$$

$$m_2 = 19 \text{ per cent}$$

Where the values of DB are thus obtained for different pH values, they are found to be higher than the values of DB of the dissociation curve

Since the values of y do not appear to be directly proportional to the values of DB in the dissociation curve, we may look for another relationship If we plot the values of $\frac{\text{per cent DB in external film}}{y}$

(at varying pH values of the external solution) against the per cent of DB in the film (z), we get a line which is fairly straight, as shown

in Fig 4 This indicates a relation corresponding to Langmuir's¹⁴ equation for adsorption,

$$y = \frac{m n z}{1 + m z}$$

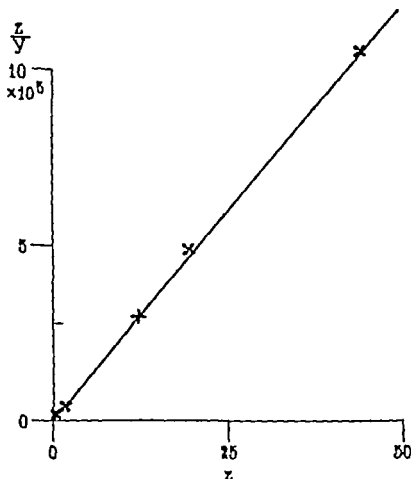


FIG 4 Graph showing that Langmuir's equation $y = \frac{m n z}{1 + m z}$ (in which $n = DB$) may be applied to the process of the exit of the dye since the graph is a straight line. Ordinates represent $\frac{z}{y}$, and the abscissae represent z .

where y is the substance adsorbed by a fixed concentration of an adsorbent, z is the concentration of the solution at equilibrium, and m and n are constants

This might be regarded as indicating that the velocity constant (k) of the exit of the dye depends on the value of y which represents the

¹⁴ Langmuir I. *J. Am. Chem. Soc.* 1918 xl 1368.

amount of DB adsorbed by the protoplasmic surface from the film of external solution just outside the surface. But the applicability of this equation does not necessarily mean that we have to do with adsorption. For example, as Hitchcock¹⁶ has pointed out, a similar relation applies if we have to do with a reversible chemical reaction where one of the reactants has a constant value.

It may be objected that if y represents the amount of DB adsorbed at the surface it will not be a constant fraction of $a - x$ during the entire process of exit of the dye but will be relatively greater during the latter part of the process. From the results of calculations which neglect this factor it is evident that it is not one of sufficient importance to effect any material change in the calculations here given.

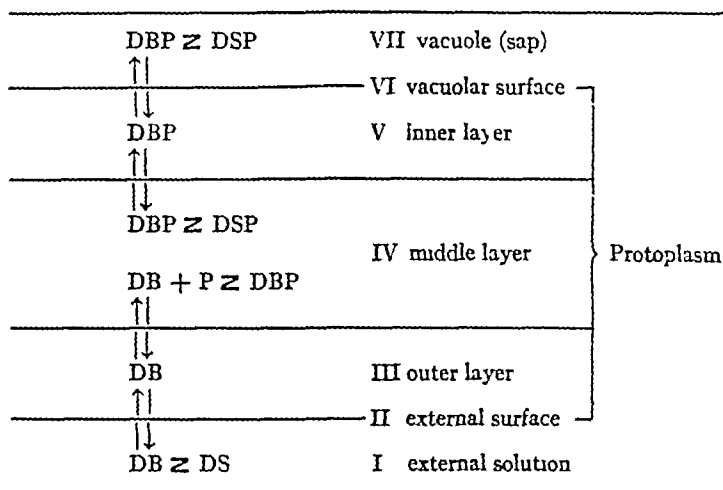


Diagram B The cell wall is omitted for convenience

If the surface forces constrain the molecules of DB so that they are not free to diffuse we meet with a difficulty. This difficulty would disappear if a constant fraction of the molecules is so constrained since that would merely lead us to divide the values of y by a constant factor.

The writer does not wish to lay any emphasis upon the fact that adsorption might possibly explain the relations observed but prefers to give the formula as a purely empirical one leaving the interpretation to future research.

¹⁶ Hitchcock, D. I., *J. Gen. Physiol.*, 1925-26, viii, 61

The preceding discussion of diffusion applies whether DB is in the form of undissociated molecules or ions

Let us now consider the hypothesis that the rate of penetration and likewise of exit of dye from the vacuole is controlled by a chemical combination between DB and a constituent of the protoplasm. For this purpose we may modify Diagram A to conform to Diagram B or C

Equilibrium between the two forms of dye is designated by the sign \rightleftharpoons and diffusion by arrows \rightleftharpoons . The entire mechanism represents a reversible process. Let us first take up Diagram B. DB can pass through III but DS cannot. As DB enters IV, it reacts with P, a protoplasmic constituent to form DBP. This form of dye compound, DBP, enters into equilibrium (according to the apparent dissociation constant) with another form of dye compound, DSP, which may represent a tautomere, or a complex compound. The ratio of $\frac{DB}{DS}$ and that of $\frac{DBP}{DSP}$ in IV depends on conditions in the protoplasm (pH value, solubility, etc.). DB, DS, and DSP are unable to pass through V, while DBP can pass through V but not through III. When DBP enters the vacuole it establishes an equilibrium with DSP, the ratio of $\frac{DBP}{DSP}$ being dependent on conditions in the sap, so that as long as the latter remain unchanged this ratio remains constant. The concentration of DBP in the sap is dependent on its concentration in the protoplasm, and on the concentrations of DB in all the parts described in the diagram. Thus, if the concentration of DB in III diminishes by its exit from III to I then DB in IV decreases by its exit from IV to III, thus resulting in a decrease in DBP which in turn causes a corresponding amount of DBP to diffuse out from VII to IV.

We may consider a cell of *Nitella* in a solution as representing a heterogeneous system consisting of at least three phases: (1) the external solution, (2) the protoplasmic layer, and (3) the sap in the vacuole. If we venture to suppose that the protoplasm has non aqueous layers at its outer and vacuolar surfaces, we shall consider the system to be composed of at least five phases.

The relation of the reaction $DB + P \rightleftharpoons DBP$ in the protoplasm to the DB in the external solution may be made clearer if we consider the

hydrolysis¹⁷ of an ester in hydrochloric acid when the ester is distributed between HCl and benzene. As fast as the ester is hydrolyzed in hydrochloric acid, more ester passes in from the benzene. The rate of hydrolysis is controlled by the distribution coefficient, C , of ester between hydrochloric acid and benzene, since the lower the value of C the less ester diffuses from benzene to the hydrochloric acid in a given time. The equation for this process resembles that for a unimolecular reaction in a homogeneous system. The only difference is that this equation contains the correction for the partition coefficient C .

Thus in the case of the reaction $DB + P \rightleftharpoons DBP$, the rate may be assumed to be dependent on the amount of DB that passes into the protoplasm. If the concentration of DB in the external solution is raised, more DB will enter the protoplasm in a given time, and this will increase the rate of reaction. Exit of the dye from the protoplasm may also be explained on this basis. If there is no DB outside the cell, DB will come out of the protoplasm, and with the decrease in the concentration of DB in the protoplasm, the reaction $DBP \rightarrow DB + P$ will proceed faster, DB thus formed will continue to come out until there is no DBP in the protoplasm. But if the DB which comes out is not at once removed, a certain amount will diffuse back into the protoplasm, so that in a given time the decrease in the concentration of DB in the protoplasm will be less than when there is no DB outside. This will correspondingly retard the process $DBP \rightarrow DB + P$, and hence diminish the rate of exit of DB.

So far as the relation of the reaction $DB + P \rightarrow DBP$ in the protoplasm to the DBP in the sap is concerned, the same explanation will hold. With an increase in the concentration of DBP in the protoplasm more DBP will diffuse into the sap. If DBP in the protoplasm decreases, on the other hand, DBP will tend to come out of the vacuole into the protoplasm. The rate of the reaction will depend on the concentration of DBP in the protoplasm. If for example the concentration of DBP in the sap is increased, causing a decrease in the amount of DBP diffusing out of the protoplasm into the vacuole, the concentration of DBP in the protoplasm will increase. This increase will retard the reaction $DB + P \rightarrow DBP$. Thus the rate of reaction $DB + P \rightarrow DBP$

¹⁷ Goldschmidt, H., and Messerschmidt, A., *Z. physik. Chem.*, 1899, xxxi, 235..

is controlled by the concentration of DB and DBP in the protoplasm. Increase in DB will hasten the reaction $DB + P \rightarrow DBP$, while increase in DBP will retard it. The concentration of DB in the protoplasm depends on the amount of DB that enters or goes out of the protoplasm at a given time, and hence on the velocity of diffusion of DB through II or III in the diagram. The concentration of DBP in the protoplasm depends on the amount of DBP that goes out of the protoplasm into the vacuole, and the amount of DBP that enters the protoplasm from the vacuole, at a given time, and hence the rate of diffusion of DBP through V or VI in the diagram, B. It must be added here that the concentrations of DB and DBP are obviously interdependent.

In view of the fact that the time curve for the hydrolysis of ester in hydrochloric acid, as described above, follows an equation similar to that of an irreversible unimolecular reaction in a homogeneous system, it is not surprising that we find in the case of penetration of dye into *Nitella* the unimolecular time curves for a homogeneous system.

Thus the analysis of the time curve of the exit of the dye may be made in this case by the use of the same equation as in the case of diffusion

$$\frac{dx}{dt} = k_1(a - x) - (k_2 y)$$

where a denotes the concentration of DB at the start in the protoplasm, $a - x$ the amount left combined with protoplasm at time t , and y the amount of DB in the film of liquid just outside the external protoplasmic surface. The presence of DB in the film will cause some DB to diffuse back into the protoplasm, and thereby retard the decrease of DB in the protoplasm. This retards the rate of the reaction $DBP \rightarrow DB + P$ and hence it retards the exit of the dye from the vacuole.

What we actually measure is the concentration of the total dye (DBP and DSP) in the sap, and the value of $a - x$ is taken from the amount of the total dye in the sap at equilibrium. This method is justified since we are interested primarily in the relative values, and since we assume that the amount of the dye in the sap has a definite ratio to that of the dye in the protoplasm.

Another method of explanation is the following, as described in Diagram C

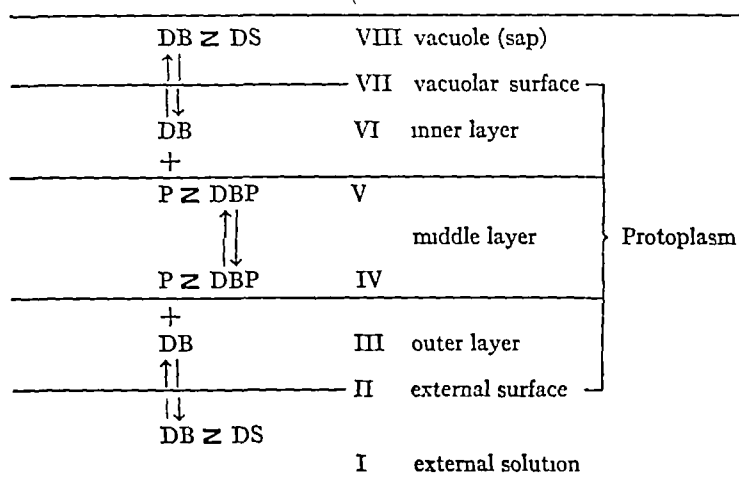


Diagram C The cell wall is omitted for convenience

DB can pass through III and VI. As DB enters at IV (the boundary between the outer layer and the middle layer) it combines with P of the protoplasm to form a complex compound DBP. DBP now diffuses from IV to V (the boundary between the middle layer and the inner layer) and DB is given off at V to VI. DB now diffuses into VIII. In I, II, VII, and VIII DB is in equilibrium with DS. The rate of $DB + P \rightarrow DBP$ is controlled by the concentration of DB and DBP in the protoplasm, and the concentrations of these substances are dependent on the amount diffusing in and out of the protoplasm in a given time. It is hardly necessary to undertake a detailed description of this diagram, as it closely resembles Diagram B. The only important differences are that the nature of the dye in the vacuole is not changed in this case, and that the reaction of $DB + P \rightarrow DBP$ in the protoplasm takes place at the boundaries IV and V. The latter may bring in complications to such an extent that we may have no justification for using an equation for a homogeneous reaction. Since so little is known in regard to this, the investigation of this question will be left to the future.

The experimental results thus far obtained do not show conclusively which one of the theories represents the mechanism. It may be possible that though there are reactions taking place between the dye and the protoplasmic constituents, the final result in both entrance of dye into and exit from the vacuole is dependent on the diffusion (see Section I) of the dye (see Foot-note 13).

A rough analogy to the passage of dye may be found in the case of entrance and exit of water into and from a reservoir, where the rate of inflow and outflow of the water depends on the conditions at the entrance and exit, and not on the conditions in the body of water between these two points

V

Rate of Exit When the pH Value of the Sap Is Changed

The following experiments were carried out to determine if the theory thus proposed is supported by the observations on changes in the rate of the exit of the dye when the pH value of the sap is varied.

One lot of cells was placed in $M/150$ borate buffer solution at pH 8.5 containing $8.6 \times 10^{-4} M$ cresyl blue and $0.005 M NH_4Cl$, at the end of 5 minutes there was $8.6 \times 10^{-4} M$ dye in the sap. The cells were then removed, wiped with a damp cloth, and placed in an $M/150$ phosphate buffer solution at pH 6.5 containing no dye. After 2 minutes, the concentration of the dye in the sap was found to be $2.6 \times 10^{-4} M$.

A second lot of cells was placed in $0.005 M NH_4Cl$ at pH 8.5 $M/150$ borate buffers at the end of 5 minutes the pH value of the sap had increased from pH 5.6 to 6.9. The pH value of the sap remained at 6.9 when such cells were placed in a buffer solution at pH 6.5 for 2 minutes.

A third lot of cells was placed in $M/150$ borate buffer solution at pH 8.5 containing $8.6 \times 10^{-4} M$ cresyl blue at the end of 45 seconds there was $8.6 \times 10^{-4} M$ dye in the sap. The cells were now removed, wiped with a damp cloth, and placed in an $M/150$ phosphate buffer solution at pH 6.5 containing no dye. After 2 minutes the concentration of the dye in the sap was determined and was found to be $5.9 \times 10^{-4} M$.

In all cases the experiments were carried out at $25 \pm 0.5^\circ C$, and the solutions were constantly stirred and changed.

From these experiments it may be concluded that the rate of the exit of the dye from the cell sap is increased by presence of NH_3 in the sap which increases the pH value of the sap. Whether this increase in the rate is due to the increase in the pH value of the sap, or to the possible presence of NH_3 and consequent increase in pH value in the protoplasmic layer or to NH_3 adhering to the cell surface, the writer is at present unable to determine.

The above observation is in agreement with the theory since the increase in the pH value would increase the concentration of DB in the sap and hence increase the concentration gradient, but in view of the fact that no appreciable changes in the pH value of the sap may be brought about without an injury to the cells, such a conclusion must necessarily be made with reserve. Furthermore, the fact that the rate of penetration is decreased,¹⁸ while that of the exit of the dye is increased, when NH_3 enters the sap, does not prove that the dye enters the cell only in the form DB. As already suggested by the writer, in case DS enters,¹⁹ the rate of penetration²⁰ may very well be decreased by the competition between the DS and the aqueous NH_3 for the substances (*viz* salt of proteins or weak acid) in the protoplasm which may take place as NH_3 and DS enter the cell. The presence of aqueous NH_3 then would hasten the exit of DS from the cell, if the affinity of NH_3 for the cell substance is greater than that of DS.

SUMMARY

Experiments on the exit of brilliant cresyl blue from the living cells of *Nitella*, in solutions of varying external pH values containing no dye, confirm the theory that the relation of the dye in the sap to that in the external solution depends on the fact that the dye exists in two forms, one of which (DB) can pass through the protoplasm while the other (DS) passes only slightly. DB increases (by transformation of DS to DB) with an increase in the pH value, and is soluble in substances like chloroform and benzene. DS increases with decrease in pH value and is insoluble (or nearly so) in chloroform and benzene.

The rate of exit of the dye increases as the external pH value decreases. This may be explained on the ground that DB as it comes out of the cell is partly changed to DS, the amount transformed increasing as the pH value decreases.

The rate of exit of the dye is increased when the pH value of the sap is increased by penetration of NH_3 .

¹⁸ McCutcheon and Lucke (see Foot-note 9) believe that the decrease in the rate of penetration of a basic dye into *Nitella* with an increase in the pH value of the cell sap is a direct disproof of the theory that the dye combines with a protein in the cell.

¹⁹ Irwin, M., *J. Gen. Physiol.*, 1925-26, ix, 235.

²⁰ When the pH value of the sap is decreased by an entrance of acetic acid the rate of penetration of dye is either increased or decreased, depending on the condition (probably of the protoplasm) of the cell.

FURTHER STUDIES ON THE INHIBITION OF CYPRIDINA LUMINESCENCE BY LIGHT, WITH SOME OBSERVATIONS ON METHYLENE BLUE

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In a previous paper (1924-25) I have shown that a filtered luminescent mixture of *Cypridina* luciferin and luciferase has its luminescence suppressed (inhibited) by light from a carbon arc in 2 or 3 seconds with 15,000 foot candles illumination. The action of the light is upon the luciferin and not upon the luciferase. I have reported the suppression as partially reversible, the luminescence returning slightly in the dark, but am now inclined to regard this effect as apparent, due to better dark adaptation of the eyes. The inhibiting wave lengths are in the blue violet region, $\lambda = 46\mu$ to $\lambda = 38\mu$. Consequently one finds no suppression of luminescence by red, orange, yellow, or green light even after prolonged exposure.

The present paper deals with three further aspects of inhibition of luminescence by light, namely (1) photodynamic action of dyes (2) influence of oxygen, (3) influence of H ion concentration on the inhibition.

1 *Photodynamic Action of Dyes*—As the dye sensitization of many photochemical reactions is well known—notably that of the photographic plate by dicyanin, pinacyanol, erythrosin etc., and the photodynamic action of acridine, eosin, etc., on living tissues, enzymes, and antibodies—it is not surprising to find a similar effect of dyes on the inhibitory action of light on *Cypridina* luminescence.

I have said that inhibition is brought about by blue (46 to 38 μ) light but not by red, orange, yellow, or green light. However, if we add to the luciferin-luciferase mixture one of a number of dyes, then we find that red, orange, yellow, or green light will inhibit the luminescence in a few seconds. The wave length of light which will inhibit

Cypridina luminescence in the presence of sensitizing dye depends, of course, on the position of the absorption band of the dye, only those wave-lengths inhibiting which are absorbed. The converse is not necessarily true, that if a dye possesses an absorption band it is a photosensitizer with respect to that light. There may be or may not be sensitization.

A number of dyes have been tested by a student of mine, Mr. A. Hunsberger, Jr. The method is this. In a dark room, light from a carbon arc in a dark house passes through 6 cm. of water and is condensed to a small beam which strikes the middle portion of a narrow test-tube containing the luminescent mixture of *Cypridina* luciferin and luciferase. The light beam can be colored by light filters and cut off instantly by a photographic shutter. The luminescence of the exposed area of the test-tube is then compared with the non-exposed regions above and below.

Wratten gelatin filters were used to obtain light of a known range of wave-lengths. As the percentage transmission of the filters varies, some being much denser than others, it is impossible to select filters that will permit equal amounts of nearly monochromatic light to pass. In fact I have selected filters of high transmission which begin to absorb strongly at some definite wave-length (No. 15, 22, 29, 88) or those with broad transmission bands (No. 61) rather than the denser monochromatic filters, in order that the exposure need not be too long. 15 seconds was selected as a convenient time.

The filters are

No. 61 green,	over 10 per cent transmission	50 to 57 μ
" 15 yellow,	" 10 " " "	52 " 70 μ and beyond
" 22 orange,	" 10 " " "	55 " 70 μ " "
" 29 red,	" 10 " " "	62 " 70 μ " "
" 88 infra-red,	no visible transmitted except 5 per cent at	70 μ

The dyes tested are given in Table I.

Allowing for the unequal transmission of the filters and the widening of the absorption bands of the dye with increase in concentration, there is undoubtedly an agreement with the rule that the dyes sensitize only for that wave-length of light which they absorb.

It can also be shown that the inhibiting action of green light in pres-

ence of eosin is upon the luciferin and not the luciferase, just as in the case of violet wave lengths acting without sensitizer.

I think we may predict with fair certainty that Ctenophores the inhibition of whose luminescence by light is so well known, will also be sensitized by photodynamic dyes.

2 *Influence of Oxygen*—It must be recalled that luminescence only occurs if luciferin, luciferase, and oxygen are together in solution, but that luciferin will oxidize without luminescence if luciferase is absent.

It seems most probable, therefore, that light acts by causing rapid

TABLE 1.

Dye	Structure.	Absorption in weak and stronger sol.	Photosensitization in 15 sec.
Fluoresceine		$\mu\mu$ 475-502 450-505	Negative.
Eosin.	K tetrabromfluoresceine.	500-530 480-540	Green yellow
Erythrosin	K or Na tetraiodofluoresceine.	500-530 480-560	"
Rose bengale.	K or Na 4 Iodo 2 chlorfluoresceine.	530-550 480-560	" "
Cyanosin	K methyl ether of 4 Br 2 Cl fluor esceine.	500-545	" "
Acridine or aniline red E 103		480-530 430-570	" "
Methylene blue.	Tetramethyl thionin HCl.	650-680 560-680	Yellow orange red.

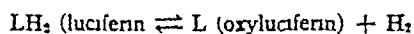
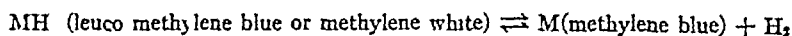
oxidation of luciferin without luminescence. Consequently the area exposed to light, of a luminescent mixture of luciferin and luciferase in a test tube will not luminesce so brightly because some of the luciferin has been photochemically oxidized. This view can be tested by completely exposing luciferin solutions to light in absence of oxygen (by evacuation or bubbling of pure hydrogen), with a control tube illuminated in presence of oxygen, and then mixing both tubes with luciferase. The luciferin exposed in absence of oxygen gives a bright light, while the control in presence of oxygen gives no luminescence or a very faint one. There is no inhibition in absence of oxygen.

I find also that the photosensitive dyes will not sensitize the inhibition of luminescence from luciferin in green or yellow light in absence of oxygen. In this respect the phenomenon agrees with photodynamic dye action in tissues. There is no poisonous action of eosin on enzymes or organisms in light in absence of oxygen (von Tappeiner, 1909).

The *dye* simply acts by making wave-lengths effective which would not be effective in its absence. The *light* acts by rapidly oxidizing luciferin.

The manner in which the photosensitizers make wave-lengths photochemically effective is not well understood. Perhaps the first question to be asked is whether the sensitizer undergoes any change. We may suppose the eosin to oxidize the luciferin in presence of light, itself undergoing reduction to a leuco body. Accordingly I have exposed mixtures of luciferin and eosin to white light (8600 foot candles) and also to green light, for from 4 to 7 minutes, but have never observed any indication of the decolorization of eosin, although it is known that eosin is affected by light (Gros, 1901). There is also no indication of the decolorization of methylene blue and luciferin exposed to white light (8600 foot candles) for 4 minutes, although the oxidative action of the light on luciferin is greatly increased by the presence of these dyes. If any change occurs in the dye it involves no color change or is momentary. Under proper conditions, however, methylene blue is affected by light, as described below.

3 *Influence of H Ion Concentration*—I have often compared luciferin to leuco methylene blue and its oxidation to the oxidation of leuco methylene blue with formation of the blue dye. Expressed as a reaction the change would be



In view of the effect of light on luciferin oxidation it is interesting to note that methylene white oxidation is affected by light also. This was observed by Clark (1925), and the effect can be very nicely seen by reducing methylene blue with Zn dust and dilute acid, pouring the

colorless solution into a narrow test tube and exposing the middle portion of the colorless solution to the condensed beam from a carbon arc lamp, first passing the beam through a water layer to remove its heat. Blueing will occur in the illuminated region in a few seconds. Oxygen is of course present in this experiment, but I can confirm Clark's observation that blueing will occur *in absence of oxygen* after reduction by platinized asbestos and hydrogen. *However, the solution must be acid.* After removal of oxygen and reduction by $\text{Na}_2\text{S}_2\text{O}_4$, blueing of methylene white in light will also occur, provided the solution is acid enough and that not too much $\text{Na}_2\text{S}_2\text{O}_4$ has been added. Neutral and alkaline solutions will not turn blue in light under the same conditions. There seems to be a shift in equilibrium of the methylene blue \rightleftharpoons methylene white system toward the side of oxidation in the light.

If methylene blue is reduced by Pt asbestos and hydrogen in two tubes, one of $\text{M}/50$ HCl , the other of $\text{M}/50$ NaOH , and the tubes shaken slightly to dissolve a little oxygen it can be easily observed that the methylene white oxidizes much more quickly in the alkaline tube, which becomes blue as compared to the acid tube, only faintly bluish—a well known phenomenon. On now exposing the two tubes to a beam of light, there results a blue band in the acid medium and a colorless band in the alkaline medium. We have acceleration of oxidation in acid and of reduction in alkali in light. I have observed the same thing when NH_4SH or H_2S is used as reducing agent and also when $\text{Na}_2\text{S}_2\text{O}_4$ is employed.

If just enough $\text{Na}_2\text{S}_2\text{O}_4$ is added to decolorize methylene blue in $\text{M}/50$ HCl and the colorless tube is exposed to light, blueing will occur, but if a little more $\text{Na}_2\text{S}_2\text{O}_4$ is added no blue band occurs in light. However, upon shaking with air until some of the $\text{Na}_2\text{S}_2\text{O}_4$ is removed by oxidation, a blue band now appears in light. Reducing with $\text{Na}_2\text{S}_2\text{O}_4$ in an alkaline medium (Clark and Lubs buffer, $\text{M}/20$ H_3BO_3 , KCl , NaOH , $\text{pH} = 10$) and exposing to light, we observe no change, but if shaken with air till partly blue and then exposing to light, a colorless band appears.

This colorless substance is methylene white and not a colorless oxidation product of methylene blue, because by thorough shaking with air the blue color will return again completely.

We may sum up the behavior of methylene blue in light as follows Methylene white in presence of reducing agents will turn blue in absence of oxygen if the solution is acid enough but not in neutral or alkaline solutions In presence of some oxygen and reducing agent,¹ acidity favors the change to blue (oxidation) while alkalinity favors the change to colorless (reduction) Without reducing agent, methylene blue will be rendered colorless by light slowly in fairly alkaline solution ($M/50$ NaOH) but not in $M/10$ Na_2HPO_4 ($pH = 9$) or in distilled water

Indigo carmine does not behave like methylene blue under the influence of illumination

If the luciferin-oxyluciferin system is to behave like methylene blue, we should expect inhibition of luminescence in light in acid medium containing some oxygen and reducing agent¹ (like $Na_2S_2O_4$) Under these conditions oxidation to oxyluciferin would be favored and less luciferin remain to luminesce with luciferase Hence a dark band should appear in a luminescent tube after illumination On the other hand, in alkaline medium a luminescent band should appear after illumination, since reduction would be favored and more luciferin accumulate in the previously illuminated region

However, I have been unable to observe a more luminescent band after illumination in solutions of any reaction If luciferin is prepared in a series of buffer solutions and a little luciferase added we get the following results in light-exposed and dark regions of the tube No reducing agent is present

Buffer solution and pH	Luminescence in dark.	Luminescence in light.
$M/20$ K H phthalate = 4	Very faint	Very slowly inhibited
$M/20$ K H phthalate, NaOH = 5.6	Faint	" " "
Sea water = 8	Good	Rapid inhibition
$M/20$ H_2BO_3 , KCl, NaOH = 9	Faint, fading quickly	" "
$M/20$ " " " = 10	" " "	" "

It will be noted that inhibition, indicating oxidation of luciferin, always occurs no matter what the reaction, acid or alkaline

If $Na_2S_2O_4$ is now added to the above tubes to remove the oxygen

¹ O⁻ its oxidation product

the luminescence disappears and exposure to light in absence of oxygen never causes luminescence to return. If the tubes are shaken slightly to dissolve oxygen, luminescence will return and then exposure to light gives the same results as recorded in the table in the absence of any reducing agent. Light exposure always results in inhibition which is more rapid the more alkaline the medium and is also more rapid in presence of the $\text{Na}_2\text{S}_2\text{O}_4$ (or its oxidation products) than previously. It is as if the oxidation products of $\text{Na}_2\text{S}_2\text{O}_4$ accelerated the effects of light, as they do in the case of methylene blue. I have never observed a more luminescent band in the region previously exposed to light.

We see that the behavior of luciferin in light is only in part similar to that of methylene blue. One always obtains acceleration of oxidation of luciferin by light and not acceleration of reduction under the same conditions (alkaline medium) necessary for the phenomenon in methylene blue. Perhaps it is pushing the analogy too far to expect that the methylene white—methylene blue and the luciferin—oxyluciferin systems will behave in exactly the same way after exposure to light.

SUMMARY

1 Eosin, erythrosin, rose bengale, cyanosin, acridine, and methylene blue act photodynamically on the luminescence of a *Cypridina* luciferin-luciferase solution. In presence of these dyes inhibition of luminescence, which without the dye occurs only in blue-violet light, takes place in green, yellow, orange, or red light, depending on the position of the absorption bands of the dye.

2 Inhibition of *Cypridina* luminescence without photosensitive dye in blue violet light, or with photosensitive dye in longer wavelengths, does not occur in absence of oxygen. Light acts by accelerating the oxidation of luciferin without luminescence. Eosin or methylene blue act by making longer wave-lengths effective, but there is no evidence that these dyes become reduced in the process.

3 The luciferin-oxyluciferin system is similar to the methylene white-methylene blue system in many ways but not exactly similar in respect to photochemical change. Oxidation of the dye is favored in acid solution, reduction in alkaline solution. However, oxidation

of luciferin is favored in all pH ranges from 4 to 10 but is much more rapid in alkaline solution, either in light or darkness. There is no evidence that reduction of oxyluciferin is favored in alkaline solution. Clark's observation that oxidation (blueing) of methylene white occurs in complete absence of oxygen has been confirmed for acid solutions. I observed no blueing in light in alkaline solution.

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TEMPERATURE CHARACTERISTICS FOR DURATION OF AN INSTAR IN CLADOCERANS

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I.

The analysis of growth phenomena through determinations of their relations to temperature requires measurements of the rates of development within stages morphologically well defined and exhibited by individuals genetically uniform and comparable. The duration of an adult instar in Cladocera parthenogenetically produced within a single clone is in several respects a very suitable object for such measurements. It is not altogether clear, however, whether the incidence of ecdysis is directly determined by the growth of the female, or by the full development of her young which ecdysis liberates. It is proposed to investigate this point more fully in subsequent experiments, but the latter interpretation is probably the correct one, for mothers about to release young, if transferred to a considerably higher temperature, will release the young properly but they themselves become caught in the moult which is being cast. In other words the young, at the higher temperature, have reached full development and have apparently caused the mother to moult before the carapace was in proper condition to be cast. For the present it is sufficient to note that the period of growth or development utilized for measurement bears relations to temperature of a kind very closely comparable with those shown by relatively simple vital processes (Crozier, 1924-25, *a, b*), and obtained likewise for the velocity of development within a clearly delimited stage in *Drosophila* (Bliss, 1925-26).

Comparison of several species of cladocerans reared in the laboratory for many generations under similar conditions should reveal spe

cific differences, should such be present, and might be taken to suggest a means for the physiological comparison of related forms

Three species of Cladocera were employed Two of these, *Moina macrocopa* and *Simocephalus serrulatus*, belong to the family Daphnidae, the third species, *Pseudosida bidentata*, belongs to the family Sidae A few individuals of *M macrocopa* and of *P bidentata* were sent me by Dr A M Banta, both forms having been reared parthenogenetically for many generations in his laboratory *M macrocopa* was originally collected near Cold Spring Harbor, Long Island, and it occurs there in small exposed ponds from April through October The population in the ponds increases rapidly to a maximum in June and the form is found in slightly diminished numbers until the middle of October The clone of *P bidentata* that was used was started from individuals collected in Florida in February, I have not taken this form in the north *S serrulatus* was collected near Cambridge, Massachusetts, early in October In general it is a species that is quite wide spread and it may also be taken during any month of the year, usually reaching large numbers towards the last half of April and the first of May, continuing with few individuals during July and August, and reaching a maximum in October

II

In the laboratory, reproduction in all species was exclusively parthenogenetic, starting from one female and thus insuring material genotypically identical throughout The stock cultures were reared at room temperature in an especially devised culture medium (Banta, 1921) The animals used in any one experiment were usually the first or second generation descendants of one individual Such animals were reared with ample food, so that they would produce average sized to large broods, brood size being a good indication of the vigor of the stock For precise work it is necessary to study a stage of development having sharply marked beginning and end In this case one adult instar, usually the second, was chosen as the period to be measured The successive instars of a given animal, when reared at the same temperature, do not show any appreciable differences in length The beginning of an adult instar is marked by the release of active young daphnids from the brood chamber of the mother, and the

end of the instar is marked by the release of the succeeding brood of young. In *M. macrocopa* the release of the young, the moulting of the mother, and the passing of a new clutch of eggs to the brood chamber occupies from 2 to 6 minutes at room temperature. The time for this series of events is slightly more variable in *P. bidentata*, but not so variable as in *S. serrulatus* in which the time from release of young to egg laying varies from a few minutes to half an hour in extreme cases.

As the adult females to be tested were nearing the end of an instar they were transferred to individual bottles and placed in constant temperature cabinets. A bottle of food was placed beside a bottle containing a female. The termination of an adult instar, that is, the appearance of young in the bottle, is foreshadowed by the darkening and coalescing of the eye rudiments of the embryos. Observation of the mothers was made at frequent intervals (15 minutes to an hour, depending on the temperature), and the time of the release of the young noted. The mother was transferred to the adjacent bottle and allowed to remain there until the next brood of young was released. When it was impossible to watch for the termination of the instar in question at intervals of an hour or less, those mothers that probably would soon liberate young were examined microscopically and an estimation made of the probable time of release of her young (based upon the condition of the eye pigment of the embryos). This estimated time was further checked by observing the developing eggs of the next brood and noting the stage of segmentation of the egg. If the elapsed time was too long the animal was of course discarded.

Four temperature cabinets were used in each of which the temperature to be maintained could be raised or lowered at will. The cabinets were heated by carbon filament bulbs controlled by a mercury thermostat. The different regions (shelves) of the cabinet varied in temperature depending on the distance from the heating unit, but the temperature of each bottle was taken directly. The extreme variation within a given bottle during an experiment was $\pm 0.5^{\circ}\text{C}$. Slightly different rates of general metabolism are found for the females producing male broods and those producing female broods (Banta and Brown, 1924-25). This has been determined for *M. macrocopa*, the male broods are released on the average later (half an hour, more or

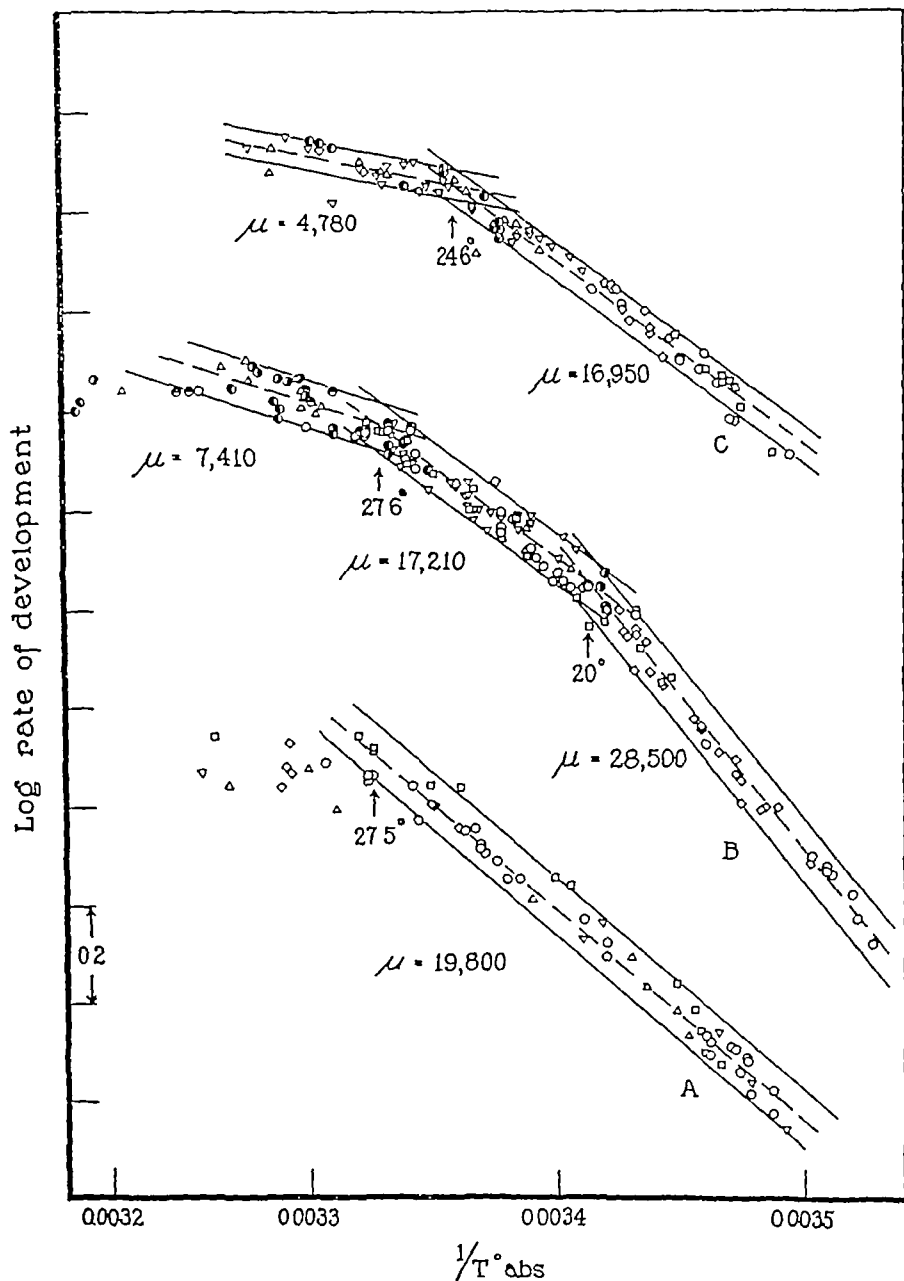


FIG 1 Graphs for rates of development plotted against reciprocals of the absolute temperatures A, *Pseudosida bidentata* B, *Moina macrocopa* C, *Simoccephalus serrulatus* Each point represents an individual animal and the different symbols in each graph denote a single experiment The values of μ are given opposite the segments of the graphs, and the critical points are indicated by arrows, with the centigrade temperatures given for these points (Rate of development = 10,000 — time in minutes, one unit on log scale = 0.2)

less) than the female broods. It has not been possible to employ numbers of animals sufficient to remove this source of variation, which would at most amount to between 1 and 2 per cent of the total observed time.

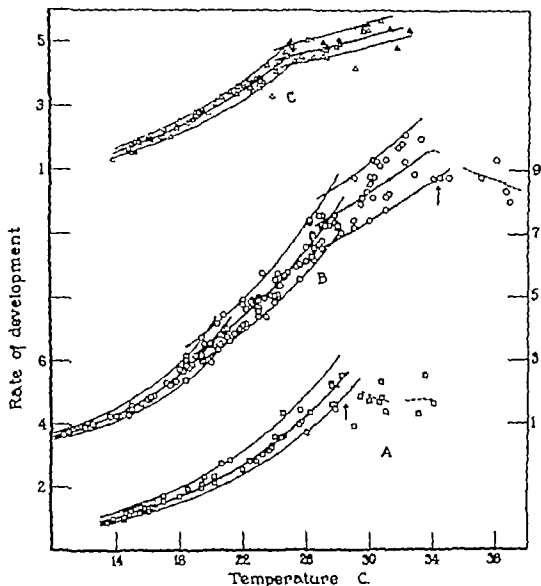


FIG 2 A, *P. bidentata* B, *M. macroscopa* C *S. serrulatus* The rates of development are plotted against centigrade temperatures and the points are entered without distinguishing individual experiments. The lighter lines and the heavier lines are transposed from those in Fig. 1

III

Fig 1 gives the graphs for the rates of development in the three species. The logarithm of the rate is plotted against the reciprocal

of the absolute temperature. Each point on the graph represents an individual animal and the different symbols in each graph denote a single experiment. The experiments were conducted at different times between January and May of the same year to examine the possibility of seasonal rhythm. It is clear that there is no secular drift due to such a cause.

The graph for *S. serrulatus* (Fig. 1, C) shows a break at 24.6°C. The slope of the line below this temperature gives a temperature characteristic $\mu = 16,950$. In the neighborhood of 15° and below there are insufficient data to determine the exact slope and the presence or absence of a break at this point. At 24°C there is a point (upright triangle) which has a lower rate. And again at 29°C there is a point (inverted triangle) which has a lower rate. In neither case was there sufficient latitude in the observations to bring these points within the range of variation of the other points on the graph. These may be individuals abnormal either through starvation or age. The character of the line above 32° has not yet been determined. The slope between 24.6° and 32° yields $\mu = 4,780$.

The graph for *M. macrocopa* (Fig. 1, B) is based on more animals than are the other two graphs. There are three evident breaks or critical temperatures. The first break is at 20°, the second at 27.6° and the third somewhere near 33°. The steepest slope, with $\mu = 28,500$, is found between 11° and 20°. The slope of the line between 20° and 27.6° gives $\mu = 17,210$, which agrees fairly well with that for *S. serrulatus* below 24.6°. Above 27.6° there is a distinct lessening of the slope, the value for μ dropping to 7,410. Above 33° the points become irregular and the curve flattens out into an almost horizontal position. It is interesting to note that even at 38° + the females of this species produce apparently normal young. The range of variation, as indicated by the limiting lines, is quite uniform for the different parts of the graph, being slightly greater above 27°, this may be due to the faster rate of development and the correspondingly decreased accuracy of the observations.

The graph for *P. bidentata* (Fig. 1, A) is strikingly different from the other two. This graph consists of one straight band, having the value of $\mu = 19,800$, extending from 14° to 27.5°. Beyond 28° the points scatter and the curve assumes a position approximately parallel

to the temperature axis. There is thus but one break, at 27.5°, in the graph for *P. bidentata*.

Inspection of graphs such as that for *M. macrocopa* in Fig. 1 may lead to the superficial suggestion that it is preferable or possible to draw through the plotted points a curve rather than several straight lines. The fact that it is impossible to fit a single simple curve to the graphs for *S. serrulatus* and *M. macrocopa* is perhaps shown more clearly in Fig. 2. In this figure the rate of development, that is the reciprocal of the time taken to complete an instar, is plotted against centigrade temperature. The lighter limiting lines and the heavier lines in this figure are transposed from those in Fig. 1. It is impossible to fit a single smooth curve through the points for either of these species. The points fall respectively into two and three cusps (when the irregular individuals above 33° for *M. macrocopa* are excluded). The points for *P. bidentata* form a smooth curve from 14° to 27.5°, this whole range yielding a constant value of μ .

The range of variation of course increases with an increase in temperature. It will also be noticed that the latitude of variation for *S. serrulatus* is much smaller than for *M. macrocopa* and for *P. bidentata*, in spite of the fact that the end points for an instar in *S. serrulatus* are more variable, intrinsically, than in the other two species. This would seem to indicate that the latitude of variation is specifically determined, but is not a property of the system controlled by the nature of the process which determines the temperature characteristic.

IV

A comparison of the values for μ with similar values obtained for rates of development in other arthropods shows some remarkable similarities, and may be taken to indicate a similar chemical control in the several instances. Bliss (1925-26) obtained a value for μ of 16,850 for the prepupal development of *Drosophila melanogaster*, from 16° to 25°. Crozier (1924-25 b), using data from Krogh, obtained a value of 16,850 16° to 32°, for μ in O_2 utilization of *Tenebrio* pupæ, and Orr (1924-25) obtained a value for μ of 16,800, 1° to 15°, for the O_2 consumption in the prepupa and pupa of *Drosophila*. These values are of the same order as the 16,950 obtained for *S. serrulatus* between 15° and 25° and the value 17,210 obtained for *M. macrocopa*.

between 20° and 28° The value of μ for *M macrocopa* below 20° does not correspond to the value obtained by Bliss with *Drosophila* below 16°, but it may be of the same nature (28,500) as the value 27,000 obtained from Krogh's data by Crozier (1924-25, b), below 22° in *Tenebrio* pupæ, that for *Drosophila* egg and larva between 10° and 20° and for *Drosophila* pupa at 15° to 20°, both of which gave $\mu = 27,000$ (Crozier, 1924-25, b, data from Loeb and Northrop) For *M macrocopa* the temperature range 28° to 33° yields $\mu = 7,410$, which compares favorably with the value obtained by Bliss for prepupal development in *Drosophila* between 25° and 30° (i.e., 7,100) Also it might be noted that the distribution of the temperature characteristics, with the exception of 4,780 for the upper portion of the graph for *S serrulatus*, corresponds to peaks in the frequency graph for temperature characteristics given by Crozier (1925-26, d)

The breaks in Fig 1, when rounded off to the nearest whole degree, are located at 15° (?), 20°, 25°, 28°, 30°, and 33° Crozier has pointed out (Crozier, 1925-26, c) that such critical points are usually found in the neighborhood of 4.5°, 9°, 15°, 20°, 25°, 27°, and 30° Setchell (1925) has pointed out that aquatic and land plants give definite points of critical temperature for anthesis, these points being at approximately regular intervals of 5 degrees from 5° to 30° The critical points obtaining for the three species of Cladocera thus agree in a rather remarkable way with those found for vital phenomena in general

A comparison of the three species of Cladocera used in these experiments shows some points of possible significance The temperatures for *S serrulatus* above which rate of development is relatively slower, i.e. lower value for μ , are 15° (?), 25°, and 30°, while the corresponding temperatures for *M macrocopa* are 20°, 28°, and 33° Thus *M macrocopa* is, upon the basis of rate of development at least, enabled to take advantage of increasing temperature by producing broods in quicker succession than *S serrulatus* And conversely, on a lowering temperature, *M macrocopa* is relatively more slowed up As previously stated, *M macrocopa* is typically a summer form while *S serrulatus* occurs throughout the year but in greater abundance in the spring and autumn *P bidentata* cannot be compared so directly This species maintains a constant $\mu = 19,800$ from 13° to 28° This

rate of increase with temperature is decidedly greater than that of the other two species above 20°. The distribution of *P. bidentata* is known to be southern and was studied during the winter, but its annual rhythm of abundance is not known at present

SUMMARY

1 The temperature characteristics for the rate of development during a well defined instar were determined for three species of Cladocera, and found to be of the same general magnitudes as those obtained for rates of development and of O₂ consumption in other arthropods

2 Critical temperatures were found to occur at points most frequently critical in quite diverse vital phenomena as determined by abrupt changes in the relationship between rate and temperature

3 A suggestion is made that, since the values of μ and the positions of critical temperatures obtained for the different species are not the same, some relation may exist between the occurrence of these forms in nature and their relative rates of development as controlled by temperatures.

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THE INFLUENCE OF LIGHT, TEMPERATURE, AND OTHER CONDITIONS ON THE ABILITY OF NITELLA CELLS TO CONCENTRATE HALOGENS IN THE CELL SAP

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In connection with a general investigation of the absorption and utilization of mineral elements by plants, various observations on the cells of *Nitella clavata* were previously reported.^{1,2} The primary object of the experimentation is to gain some additional insight into the fundamental processes of absorption in their relation to the nutrition of higher plants. Special attention has, therefore, been given to dilute solutions, comparable to soil solutions, and to the intake of mineral elements by various types of plant cells from the point of view of growth and metabolism, rather than that of permeability *per se*. Certain phases of the general problem which were previously outlined have now received further study and the development of a number of new methods of procedure has made it possible to obtain much more extensive and satisfactory data than heretofore. Reference to the recent work of Osterhout and his colleagues on *Valonia* and *Nitella* will be deferred until later in the discussion.

In our earlier experiments on *Nitella* certain preliminary data were obtained on the absorption of bromine. This element seemed to offer promise of being useful in studying the phenomena of absorption, since it is non-toxic or practically so in low concentrations, and is not normally present in the cell sap of these plants. At first the estimation of bromine was made by a colorimetric method, but this was not found to be satisfactory for quantitative work and it did not appear

¹ Hoagland D. R. and Davis, A. R., *J. Gen. Physiol.*, 1922-23, v, 629

² Hoagland D. R. and Davis A. R. *J. Gen. Physiol.*, 1923-24, vi, 47

that any technique so far described for determining bromine met our particular requirements. One of the writers (P. L. Hibbard), therefore, devoted considerable effort to devising a method suited to the purpose. Eventually, it was found possible to determine bromine in the presence of chlorine or iodine with the use of very small quantities of cell sap (1 cc.) and within a reasonably short time. The analytical procedure and the magnitude of the error, when this method is applied to plant sap, are discussed elsewhere.³ Considering the very minute absolute quantities dealt with, the accuracy of the results was better than might be anticipated. As a rule errors other than those connected with the analysis limited the interpretation of the data, except in those cases in which very small concentrations of bromine were present in the sap, when the percentage of error in the analytical data was unavoidably high. Nearly all analyses were made in duplicate and the experiments were also duplicated or repeated. It is believed that due care has been taken to limit the conclusions in accordance with the significance and consistency of the results, as will be further indicated in the discussion of the specific data. Fortunately, the effects we sought to demonstrate were of large relative magnitude so that they stand out quite clearly. At the present stage of development, it is extremely doubtful whether anything would be gained by any considerable refinement of the experiments, which might involve an almost prohibitive amount of labor.

Cell sap only slightly contaminated was obtained by the method of breaking individual plant cells in the manner previously described. Cells used for this purpose were very turgid and varied in length from $\frac{1}{2}$ to 3 inches. In addition to this method of obtaining sap, in a few experiments, sap was also prepared by expressing (by hand pressure) the masses of cells remaining after nearly all the large cells had been selected out. The sap prepared in this way and filtered is designated as "expressed sap" and that derived from individual cells as "cell sap." The different substances were present in the expressed sap in concentrations only about one-half those of the cell sap. Of course, there must have occurred a very appreciable dilution of the cell sap in the former case by water still adhering to the outer surfaces, even after

³ Hibbard, P. L., *Ind. and Eng. Chem.*, 1926, xviii, 57.

shaking the mass of cells vigorously, but it is also possible that the many very small cells (not more than $\frac{1}{4}$ inch in length) contained sap of lower concentration than that of the sap in the large cells. Conclusions with regard to the general relations existing between the cell sap and the external medium would have been very similar if the expressed sap alone had been considered, but the results on the cell sap are obviously more definite and convincing, and, furthermore, the expressed sap cannot give an adequate idea of the extent to which certain elements may become concentrated in the vacuole.

In many of the experiments it was found convenient to use a general culture solution to which bromide was added as desired. This solution was buffered with phosphate and had the following approximate composition

KH_2PO_4	5 milli-equivalents
$\text{Ca}(\text{H}_2\text{PO}_4)_2$	2 "
NaOH	6 "
pH.	5.0-5.4 ⁴

During the course of experiments of extended duration the initial pH value of the culture solution usually increased by several tenths, whether as a result of selective absorption or because of the lime deposits frequently adhering to the outside surfaces of the cells. Solutions of the composition stated above were not found to show any apparent toxicity within the time of the experiments. Under favorable environmental conditions, masses of cells could be kept in such solutions over periods up to 2 months in as good a condition as in tap water. Bromides in a concentration of 0.05 molar were not toxic as far as could be observed and much higher concentrations produced only very slight injury, if any. Of course, in any solution, there occurs a gradual dying off of a certain number of cells, but this was not more noticeable with the experimental solution than with tap water for the

⁴ Loss of chlorine accompanied by injury was not found to occur unless the pH value was below 4.8. The marked loss of chlorine and injury beginning at about pH 4.4 is correlated by Pearsall (Pearsall W. H. and Ewing J. *New Phytologist* 1924, xiii 1923) with the isoelectric point of the *Nitella* proteins. With regard to the pH value of the cell sap the various treatments were not found to alter appreciably the normal value of 5.2.

periods in question. In both types of media, under appropriate conditions of light and temperature, much new growth took place in the course of a month or more. After 2 or 3 months, cells kept in these solutions showed evidence of injury, but a complete culture solution

TABLE I

Comparison of Concentration of Br and Cl in Sap from Cells Exposed to Different Types of Media Containing KBr or KCl

Composition of medium.	Concentration of Br or Cl in cell sap.	Period of exposure.
	m. Eq	
Buffer solution + KBr 5.0 m.-Eq	22.7 Br	6 days continuous illumination
KBr 5.0 m.-Eq, no buffer	24.8 "	" " "
Buffer solution + KBr 5.0 m.-Eq	57.0 "	17 days continuous illumination
KBr 5.0 m.-Eq, no buffer	49.5 "	" " "
Buffer solution + KCl 5.0 m.-Eq	127.5 Cl	6 days continuous illumination
KCl 5.0 m.-Eq, no buffer	118.0 "	" " "
Buffer solution + KCl 5.0 m.-Eq	136.2 "	17 days continuous illumination.
KCl 5.0 m.-Eq, no buffer	140.5 "	" " "
Buffer solution + KBr 5.0 m.-Eq	20.5 Br	6 days daylight.
Tap water + KBr 5.0 m.-Eq	11.3 "	" " "

pH of phosphate buffer solutions + KBr and of KBr solutions 5.0–5.4 pH of tap water + KBr 7.0 or above

Other data prove the lessened absorption from tap water was, to a large extent, caused by the influence of the chlorine ions

(plus CaCO_3) containing bromide was as favorable a medium as tap water, judging by the new growth obtained during the course of a year

Later studies showed that the accumulation⁵ of Br was very similar

⁵ For convenience of discussion, we are using the word "accumulates" in the sense proposed by Osterhout, i.e. when a substance reaches a higher concentration in the sap than in the surrounding solution

whether KBr was used alone or added to the buffer solution (Table I). It may be noted that the former solutions were not completely free of traces of calcium, because of contamination from surface deposits on the cells. Therefore, no conclusion can be drawn with regard to the effect of a complete absence of calcium from the solution, but it is evident that in dilute solutions of this character, relatively high in K, the calcium added to the solution had no striking effect on the accumulation of Br, and that the use of buffer salts was not influential in determining the course of absorption.

Most of the experiments were carried out in beakers or wide mouth bottles with a capacity of 3 or 4 liters. From 100 to 125 gm of cells, drained free of excess water, were placed in 3 liters of solution. The mass of cells was previously washed thoroughly in distilled water. At the end of an experiment the cells were removed from the solution, first washed with tap water and then with distilled water, after which the sap was recovered as already indicated. Except in experiments in which complete analyses were to be made, when several thousand cells were used, each sample of cell sap ordinarily represented several hundred cells and varied in volume between 2 and 4 cc. Because of the large number of cells which each sample of sap represented, errors resulting from the variability of individual cells were reduced sufficiently to permit satisfactory comparisons of different treatments. Incidentally, it may be remarked that while the whole procedure is exceedingly tedious and time-consuming, no easier way of obtaining direct evidence concerning the composition of the cell sap has been suggested, and it is just this type of evidence which is most needed at the present time.

Temperature Effects

In this series of experiments, we desired to obtain some indication of the temperature coefficient for the absorption of Br under controlled light conditions. Two double walled baths were constructed with arrangements for flowing tap water or ice water. Heat was supplied by 100 watt lamps covered with tin foil and immersed in the water contained in the inner compartment. The lamps were connected with a mercury thermoregulator capable of regulating the temperature of the inner bath to $\pm 1^{\circ}\text{C}$. Mechanical stirrers were placed at one end of this bath.

The *Nitella* cells were contained in large beakers or jars set in the inner compartments and were illuminated by two 100 watt lights approximately 1 foot apart and suspended about 1 foot above the containers. Porcelain reflectors were used. It was found that the radiation from the lights caused the upper few inches of solution in

TABLE II
Concentrations of Br in Sap from Nitella Cells after Exposure to Bromide Solutions Kept at Different Temperatures

No of experiment.	Temperature	Concentration of Br in cell sap	Temperature coefficient (10°C)	Concentration of Br in expressed sap	Temperature coefficient (10°C.)	Period of exposure
	°C	m EQ		m EQ		hrs
1	10	4	3.5	3	2.7	6
	20	1.4		8		
2	10	2.2	2.9	1.2	2.7	25
	20	6.4		3.2		
3	10	4.3	2.9	2.7	2.6	50
	20	12.3		7.0		
4	10	4.0	2.5	1.9	2.6	52
	20	10.0		5.0		
5	10	7.1	2.3	3.6	2.4	68
	20	16.6		8.7		
6	14	12.5	2.0	6.6	2.2	72
	24	24.4		14.7		

In Experiments 1, 5, and 6 the values are averages of duplicate experiments

Phosphate buffer solutions + 0.05 M KBr Initial pH 4.8-5.1 Final pH 5.6-5.8

Continuous illumination with two 100 watt lights, suspended approximately 1 foot above the jars containing the cells. Temperatures of bath kept within $\pm 1^\circ\text{C}$, except for occasional short periods when adjustments were being made

which the cells were immersed to be several tenths of a degree higher in temperature than the body of the solution, but the increase was the same for both temperatures compared. In any case, it was evident that the temperature control was much more accurate than the possible control of other factors. In this series of experiments, the illumination was continuous and daylight was excluded

In Table II are presented results showing the concentrations of Br found in the sap of cells which had been exposed to solutions kept at temperatures 10°C apart, *i.e.* $14\text{--}24^{\circ}\text{C}$, and $10\text{--}20^{\circ}\text{C}$. It would have been desirable to have made measurements at numerous intervals of time for each temperature, but this would have involved the use of many large containers kept under definite temperature control, for which no facilities were available. However, while velocity constants could not be calculated, the data, taken as a whole, seem to indicate quite clearly that the temperature coefficient (between 2.0 and 3.0 for 10°C) for the absorption of bromine under the conditions specified is of the order of magnitude generally characteristic of chemical reactions rather than of diffusion processes, and this is the main point of interest at present.

Effect of Light on Accumulation of Br

Earlier experiments showed that the removal of Cl from dilute solutions was definitely influenced by the conditions of illumination. Several preliminary experiments also indicated that the penetration of NO_3 or of Br into the sap of *Nitella* cells was likewise accelerated under the influence of light. In the first series of the present experiments, the temperature arrangements above described were utilized. The exclusion of light, when desired, was accomplished with large beakers painted black on the outside and covered loosely over the top with black paper. The periods during which the cells were exposed to the solution containing bromide (buffer solution plus 5 milli-equivalents KBr) were relatively short but there was a marked difference between the illuminated and unilluminated cells in respect to the concentrations of Br present in the cell sap at the end of the experiment (Table III). Except in one instance, the concentrations of Br in the samples of sap obtained from the illuminated cells were from about two to four times those in the sap from the unilluminated cells.

Subsequently, numerous additional experiments were performed over longer periods of time, without the use of the temperature baths, but with such small differences in temperature between the illuminated and unilluminated cells as to be negligible for this purpose. In every case, exposure to light strikingly increased the ability of the cells to accumulate Br. In fact, when solutions of 5 milli-equivalents KBr

TABLE III

*Concentration of Br in Sap from Illuminated and Unilluminated Nitella Cells after Exposure to Bromide Solutions **

No. of experiment	Temperature.	Concentration of Br in cell sap.	Light condition	Period of exposure
	°C	m. EQ		hrs
1	10	2 2	In light.	25
	10	1 7	" dark	
	20	6 4	" light.	
	20	2 6	" dark	
2	10	4 3	" light.	50
	10	1 9	" dark	
	20	12 3	" light	
	20	3 3	" dark.	
3	10	4 0	" light.	52
	10	2 2	" dark.	
	20	10 0	" light.	
	20	3 2	" dark	

* Solutions, illumination, and temperature arrangements same as described in Table II

TABLE IV

Concentration of Br in Sap from Nitella Cells Exposed to Bromide Solutions in Continuous Darkness, with Longer Periods of Exposure

No. of experiment.	Medium.	Period of exposure	Concentration of Br in cell sap.
		days	m. EQ
1	Tap water and 50 m.-Eq KBr, in dark	5	5 0
	Same, in daylight.	5	11 2
2	KBr, 50 m.-Eq in dark	7	5 2
	Same, in daylight and artificial light.	7	19 3
3	KBr, 50 m.-Eq in dark.	5	5 0
4	KBr, 50 " " "	2	2 5
5	KBr, 50 " " "	2	1 6
	Same, in dark.	6	4 3

KBr solutions without buffer salts

These experiments were carried out at room temperature, averaging about 20°C. At the end of the longer periods in the dark, many small cells had died, but the larger cells from which sap was obtained were turgid

were employed, the concentration of Br in the sap of cells kept in the dark did not exceed that present in the outside solution, while the sap from illuminated cells subjected to similar solutions for equal periods of time contained much higher concentrations of Br. This was true of several different media containing bromide (Table IV). When media with concentrations of 1 milli-equivalent KBr were employed, there was considerable evidence that the concentration of Br in the sap could exceed that of the solution, even under conditions of darkness, but the quantities involved were too small to admit of certainty.

It was not found possible to keep the cells in the dark for a very extended period because of injury which was accelerated by the development of microorganisms. The question then arose whether cells kept under a normal condition of alternating periods of light and darkness could concentrate Br in the sap during the periods of darkness. It appeared that this point could be tested by alternating solutions in such a manner that some cells would have access to Br only during the periods of illumination and other cells only during the periods of darkness. Accordingly, two experiments of this type were carried out. In the first experiment, the periods were divided approximately into 12 hours of illumination and 12 hours of darkness. At the end of each period, a transfer of solutions was made, after washing the cells very thoroughly with distilled water. The result of this experiment (Table V) was that the sap from the cells having access to Br only during the periods of illumination contained Br in a concentration several times that of the external solution, but in the sap from the cells immersed in the bromide solution only during periods of darkness, the concentration of Br was not greater than that of the outside solution.

This experiment did not indicate that there was any residual effect of light but it was deemed advisable to make a further experiment over a longer total period of time, with 24 hour periods of illumination followed by 24 hour periods of darkness. The solutions were changed in the same way as before. Again a marked difference was found between the cells kept under the two conditions, but the sap from the cells placed in the bromide solutions only during periods of darkness had accumulated Br in a concentration significantly greater than that

of the outside solution. The concentration in the sap was also much greater than could occur with cells kept an equal time in continuous darkness. Evidently, under suitable conditions, some effect of the

TABLE V
Absorption of Br in Dark by Cells Exposed to Alternating Periods of Light and Darkness

Experiment 1		
(Approximately 12 hr periods of alternating light and darkness. Total duration 101 hrs *)		
	Concentration Br in cell sap	Concentration Cl in cell sap
	m Eq	m Eq
A Cells having access to KBr (concentration 5.0 m.-Eq) only when illuminated	18.2	102.5
B Cells having access to KBr (concentration 5.0 m.-Eq) only when in dark	3.9	116.7
Experiment 2		
(Approximately 24 hr periods of alternating light and darkness. Total duration 10 days)		
A Cells having access to KBr (concentration 5.0 m.-Eq) only when illuminated	37.8	96.4
B Cells having access to KBr (concentration 5.0 m.-Eq) only when in dark	19.0	112.5
C Cells having access to KBr (concentration 1.0 m.-Eq) only when illuminated	24.9	106.0
D Cells having access to KBr (concentration 1.0 m.-Eq) only when in dark	6.4	115.8

Values for concentrations in sap, averages of duplicate experiments. Solutions were buffered with phosphate, as in other experiments.

Experiment 1—Illumination by two 300 watt lights suspended about 1 foot above jars. Temperature $24^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$.

Experiment 2—Illumination by two 300 watt lights suspended about 1 foot above jars. In this experiment, diffused daylight supplemented the artificial illumination. Temperature $20\text{--}25^{\circ}\text{C}$.

* First period in light was 17 hrs duration.

illumination can be carried over to a subsequent period of darkness and influence the absorption or accumulation of substances. It cannot be stated at present whether or not this effect is concerned with a storage of available carbohydrates, although such a suggestion is a

natural one. We know that certain types of cells must always be confined to the indirect use of light energy through the oxidation of carbohydrates. In the case of the *Nitella* cells, the ability to store easily available sugars seems to be very limited.

The next experiment on the effect of illumination was planned for the purpose of determining how varying the periods of illumination during each 24 hours would influence the amounts of bromine concentrated in the cell sap. In this experiment, the jars containing the *Nitella* cells were placed in a glass chamber originally designed for studying the growth of wheat plants under controlled light conditions. The chamber was illuminated by six 500 watt Mazda lights (with

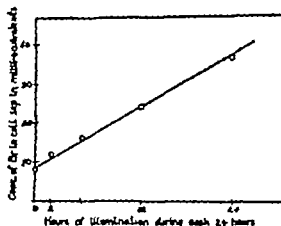


FIG. 1. Relation between number of hours of illumination and accumulation of Br in sap of *Nitella* cells. During periods of exposure, cells were illuminated by six 500 watt Mazda lights. (See text.)

metallic reflectors) arranged about the sides and ends of the chamber, and approximately 3 feet distant from the jars containing the *Nitella* cells. The temperature was controlled at 23–25°C with the aid of an air current flowing through the chamber. The light was cut off from certain cells when required by placing over the jars cylinders of heavy brown paper with loose coverings for the top. This arrangement shut out practically, but not absolutely all the light. The illuminated solutions had a slightly higher temperature, about 1°C, than the others, but the effects of varying the periods of illumination were so great that this factor was negligible in comparison.

Referring to Fig. 1, it will be observed that there was a striking increase in the concentration of Br in the cell sap with each increased

period of illumination The greatest concentration was found in the sap from cells exposed to light continuously for 3 days The concentrations of Br do not vary in direct proportion to the periods of illumination, but an approximately straight line is formed by plotting the hours of light against Br concentrations The total number of points is not sufficiently great to warrant an attempt at a mathematical analysis, but there can be no doubt of the influence of the length of the periods of illumination This conclusion is in accord with our previous observations on the removal of Cl from dilute solutions

With regard to the possible relations existing between quality or intensity of light and the accumulation of Br, it will be necessary to make additional experiments For the present, it may be noted that, in general, the most rapid accumulation occurred where the intensity of light was greatest One experiment was carried out for the specific purpose of comparing two intensities of light, maintaining the temperature the same (approximately 20°C) in both cases within 2 of a degree It was found that doubling the light (100 and 200 watts) increased the absorption 30 per cent as an average of duplicate experiments which were in close agreement In another test, the results indicated that yellow light (potassium chromate solution filter), was at least as efficient as the white light from Mazda lamps Blue light (alkaline copper solution filter) was less effective, but this observation has no necessary significance since the total energy values of the different lights are not known

The Effect of Toxic Agents

The ability of *Nitella* cells to concentrate various substances in their cell sap is apparently bound up with the processes of growth or metabolism Toxic agents might, therefore, be expected to interfere with such a concentrating action We have made several experiments which seem to support this assumption (Table VI) Different toxic substances were added to the bromide solutions and the concentration of Br in the cell sap determined as in the other experiments In some cases, many small cells were killed by the toxic substances, but the samples of sap used for the analysis were, of course, obtained from those large cells which still remained turgid at the end of the period of exposure It is reasonable to suppose, however, that these

cells also suffered some injury. Prolongation of the treatments would have resulted in the death of all the cells.

The injury has evidently caused, or been accompanied by, a decreased ability on the part of the cells to concentrate bromine in the

TABLE VI
Effect of Toxic Agents on Concentration of Br in Cell Sap

No. of experiment.	Treatment.	Concentration of Br	Period of exposure.
1	None	28.8	3 days continuous illumination.
	1 p.p.m. KCN	29.0	
	10 " "	18.2	
2	None.	19.7	5 days daylight.
	Chloroform.	12.5	
3	None.	13.5	4 " "
	10 p.p.m. KCN	14.1	
	20 " "	8.7	
4	None.	22.6	4 " "
	Chloroform.	9.4	
	Ether	14.7	
	20 p.p.m. KCN	12.5	
	Thymol excess.	12.5	

Phosphate buffer solutions containing 5.0 milli-equivalents KBr. 1 cc. of ether or chloroform added to 3 liters of solution. Treatment with ether repeated several times. Additional chloroform added once in Experiment 4. In Experiment 4 results are averages of duplicate experiments, except for last treatment. The differences between duplicates were much smaller than those between the control and the treated cells.

cell sap. Tröndle,⁶ by indirect methods, has reached a somewhat similar conclusion with the use of various salts. While, in the case of anesthetics, the electrical resistance of certain plant cells may be increased over a limited period of time,⁷ any marked injury generally involves increased permeability, as, for example, in the experiments

⁶ Tröndle, A. *Biochem. Z.* 1920 cxii, 259.

⁷ Osterhout W. J. V. Injury recovery and death in relation to conductivity and permeability. Monographs on experimental biology, J. B. Lippincott Company Philadelphia and London, 1922.

of Osterhout⁸ and Brooks⁹ on *Nitella* cells. In our view, such increased permeability may be accompanied by a decreased ability to concentrate substances in the sap. This important distinction will be referred to again in the later discussion.

The Exchange of Substances between Cell Sap and Culture Solution

Before giving further consideration to the accumulation of Br in the cell sap, it is necessary to inquire into the possibility of an exchange of

TABLE VII
Exchange of Br and Cl between Cell Sap and Culture Medium

Composition of original solution	Found in solution after contact with cells		Period of exposure
	Br	Cl	
Experiment 1			
Phosphate buffer + KBr 50 m -Eq	<i>m</i> Eq 3 85	<i>m</i> -Eq 68	<i>days</i> 7
“ “ + KCl 50 “	44*	3 66	
“ “ + KBr 50 “	2 44	1 38	43
“ “ + KCl 50 “	90*	3 24	
Experiment 2			
Phosphate buffer + KBr 50 m -Eq	2 40	1 24	21

Cells kept at room temperature in daylight. The proportion of cells to solution was approximately 30 gm. of the former to 600 cc. of the latter.

The masses of cells were composed chiefly of small, very green and turgid cells, practically all in healthy condition, judged by appearance. No evidences of injury developed during the experiment. Similar cells kept in the phosphate solutions without Cl or Br did not lose these elements to the solution sufficiently to give a definite test with AgNO_3 .

* The cells used in these tests had previously been exposed for several months to solutions containing 0.02 M KBr.

ions between the cell sap and the culture medium. Previous work had shown that the contents of the sap did not appear to diffuse out unless the cells were injured. When masses of healthy and uncon-

⁸ Osterhout, W. J. V., *J. Gen. Physiol.*, 1922-23, v, 709.

⁹ Brooks, M. M., *J. Gen. Physiol.*, 1921-22, iv, 347.

taminated cells were immersed in a phosphate buffer solution, for example, the test for Cl in the solution was negative or only indicated a very slight trace, even after the lapse of several weeks. The same observation has been made with solutions containing sulfate or nitrate. But under exactly similar conditions, except with the substitution of bromide, very appreciable quantities of Cl were found in the external solution after from 1 to 6 weeks (Table VII), although in this time the cells presented no evidence of injury, judged by their appearance. In the course of a month, considerable new growth had occurred. The amounts of Cl lost from the cells were of such magnitude that they would have been followed by obvious signs of injury or death of the cells, had the loss occurred from cells exposed to solutions not containing bromide. Furthermore, when cells which had previously accumulated a high concentration of Br in their sap were placed in solutions containing chloride, Br entered the solution, but no appreciable amount was lost to similar solutions not containing chloride. For these reasons, it is very difficult to ascribe such exchanges to cell injury. If injury were present, it would seem that it must have been too slow to account for the effects noted. Another point to be emphasized is that Cl may be lost from cells at the same time Br is being concentrated in the sap. The studies on the Br and Cl content of the sap itself will now be described.

Accumulation of Br over Various Time Intervals

In these experiments, Br determinations on the sap were made as before. Cl was determined on the same samples and the changes in its concentration were estimated by obtaining the difference between the concentration of Cl in the sap from untreated cells and in that from similar cells after exposure to the solutions under investigation. In many cases, the differences were comparatively large and since each sample was representative of a great many cells, the values for the increases or decreases of Cl concentration determined in this way are undoubtedly significant of loss or gain of Cl by the cell sap.

We now desire to direct attention to Fig. 2 in which are plotted the changes in Br and Cl concentrations of cell sap, which occurred over a period of 40 days. The cells were kept continuously illuminated and while the temperature could only be controlled very roughly, the

average temperatures for each time interval were sufficiently alike so that no important fluctuations in the general trend of the curves is noted. It will be observed, first, that the accumulation of Br takes place in a very gradual manner and that 40 days elapsed before a condition of apparent equilibrium was attained. This slow accumulation of Br in the cell sap was very definitely accompanied by slow loss of

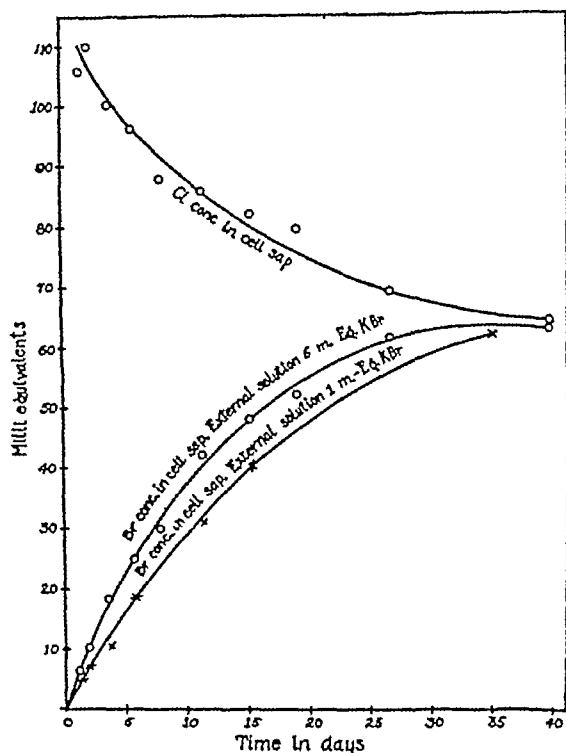


FIG. 2 Rate of accumulation of Br and loss of Cl over period of 40 days. Continuous illumination. Diffused daylight supplemented by artificial illumination by two 300 watt Mazda lights.

Values averages of duplicate or triplicate experiments, agreeing usually within 10 per cent. Error of 25 per cent may occur with very small concentrations of Br.

Cl until at the end of 40 days, the equivalents of Cl and Br present in the sap were almost equal. At this time, the masses of cells all appeared to be in a healthy condition.

While a loss of Cl took place, it is especially to be noted that the total halogen content of the sap was decidedly increased. In other

words, the equivalents of Br accumulated exceeded those of Cl lost from the cells

In this experiment, comparison was also made of solutions containing 5 milli-equivalents and 1 milli-equivalent of KBr. The two curves representing the increases in the concentrations of Br in the cell sap are not widely separated and at the conclusion of the experiment, the concentrations in the sap of the cells kept in the solution of 1 milli equivalent KBr and in that of 5 milli-equivalents KBr were almost the same. Evidently, there is no equality in the ratios of internal to external Br concentration with different concentrations of bromide present in the culture solutions. Further data pertaining to this point will be presented in a later discussion.

Recently Irwin¹⁰ has subjected her data on the accumulation of a dye by *Nitella* cells to a mathematical analysis using the formula $K = \frac{1}{t} \log \frac{a}{a-x}$, in which K is a constant, t time, a a concentration of dye in sap at equilibrium, and x concentration of dye at time t . It was thought that some interest might attach to the application of a similar formula to the data plotted in Fig. 2. (In this case, a and x refer to Br concentrations.) With the solution containing 5 milli equivalents of KBr, a fairly good agreement is found between observed and calculated values of x , having in mind the difficulties of experimentation (Table VIII). The number of observations on the solution of 1 milli-equivalent KBr concentration was too small for the purpose but a lower value of K for this solution is indicated. With regard to the rate of loss of Cl, a lower value of K was obtained than for the accumulation of Br.

While the formula applied by Irwin to the accumulation of dye may fit approximately the data for the accumulation of Br, it does not, of course, follow that the two processes are necessarily similar. There is a very striking difference in the time required for the attainment of an equilibrium condition. In the case of the dye this condition was reached in a few hours, while for Br 30 or 40 days was necessary, even under favorable light and temperature conditions. The ratio between the concentration of dye in the sap and in the external solu

¹⁰ Irwin M. *J. Gen. Physiol.* 1925-26 vol, 147

tion was found to be constant in the range studied, but such constancy of ratios is far from applying to the accumulation of Br. It was concluded that the dye very possibly combines with some organic constituent of the sap. There is no evidence that the accumulation of Br can be explained on the basis of organic combinations in the vacuole, although it may well be true that intermediate processes involve such combinations with some constituent of the protoplasm.

TABLE VIII

Accumulation of Br in Cell Sap of Nistella
(Fig. 2)

Calculated according to formula used by Irwin for accumulation of dyes

$$K = \frac{1}{t} \log \frac{a}{a-x}$$

k = constant, a , concentration of Br at equilibrium

x = concentration of Br at time t

$a = 64.6$

Br concentrations in milli-equivalents

Time	K	x observed	x calculated. $K = 0.37$
days			
1 15	0.36	6.1	6.3
2	0.37	10.1	10.1
4	0.37	18.6	18.7
6	0.37	25.8	25.8
8	0.34	30.4	31.9
12	0.39	42.8	41.4
16	0.38	48.4	48.1
20	0.38	53.4	52.9
28	0.38	63.2	58.7
40	0.36	64.6	62.5
Average	0.37		

Culture solution phosphate buffer + KBr, 5 milli-equivalents, same as used in other experiments

Other Reciprocal Relations of Br and Cl

In connection with the displacement of Cl by Br, the question arose whether cells with Cl content initially higher than that of normal sap

could accumulate Br with the same rapidity as normal cells (*i.e.* cells kept in tap water). Several experiments were made with this point in mind. During an initial period of 10 to 14 days, certain lots of cells were exposed to a buffer solution without Cl and others to a similar solution to which 5 milli-equivalents of KCl had been added. At the end of this initial period, the cells (A) which had been in the solution without Cl were transferred to a solution containing 5 milli-equivalents KBr. A portion of the cells (B) which had been kept in the chloride solution were also placed in a solution containing KBr. Another portion of the cells (C) from the chloride solution was transferred to a solution without Cl or Br. The second period of the experiment was from 5 to 9 days in length. In each experiment cells were maintained under the same light and temperature conditions. At the end of the final periods, samples of sap were obtained from the various lots of cells and determinations of Cl and Br were made (Table IX).

Attention is first called to the fact that the Cl content of the cell sap (C) could be increased from 30 to 40 per cent under the conditions described. These and other experiments suggest, however, that the accumulation of additional Cl is less rapid than the accumulation of Br. The second important observation is that the cells (B) which had previously had their Cl concentration increased accumulated a much smaller amount of Br than did the cells (A) which had a normal Cl content in the sap at the time they were placed in the bromide solution. In the latter cells, a considerable concentration of Br was attained without anything like an equivalent displacement of Cl, but the accumulation of Br by the cells with the initially higher Cl concentration was at the expense of a nearly equivalent displacement of Cl. The cells behaved as though the total halogen content could be increased to a certain point, after which accumulation of halogens occurred only as a result of exchange or displacement. The consistent data obtained in three independent experiments under different conditions, seem to warrant these statements.

Conductivity Data.

If the total electrolyte content of the cell sap can be increased, we should be able to show that increases in conductivity also occur. In order to determine the extent of such changes, a special conductivity

TABLE IX

Retarding Effect of Preliminary Accumulation of Cl on Subsequent Accumulation of Br

Culture solutions (a)	Concentration of Cl in cell sap	Concentration of Br in cell sap	Light and temperature conditions
Experiment 1			
A Period 1, no Cl or Br added " 2, KBr, 5 m -Eq	m Eq 97 2	m Eq 32 0	Period 1, 14 days, Period 2, 9 days Continuous illumination (b)
B " 1, KCl, 5 " " 2, KBr, 5 "	122 0	13 8	Same conditions
C " 1, KCl, 5 " " 2, no Cl or Br added	137 5	0	" "
Experiment 2			
A Period 1, no Cl or Br added " 2, KBr, 5 m -Eq	103 0	40 1	Period 1, 10 days, Period 2, 5 days Continuous illumination (c)
B " 1, KCl, 5 " " 2, KBr, 5 "	117 5	22 7	Same conditions
C " 1, KCl, 5 " " 2, no Cl or Br added	141 0	0	" "
Experiment 3			
A Period 1, no Cl or Br added " 2, KBr, 5 m -Eq	101 5	22 4	Period 1, 10 days, Period 2, 6 days Continuous illumination (d)
B " 1, KCl, 5 " " 2, KBr, 5 "	129 5	10 9	Same conditions
C " 1, KCl, 5 " " 2, no Cl or Br added	137 2	0	" "

All experiments carried out in duplicate, with agreement within 10 per cent of total value, except in one case. Average values given.

(a) Phosphate buffer solutions + 5 milli-equivalents KBr or KCl, as indicated. pH approximately 5.4

(b) Illumination by two 300 watt lamps placed about 2 feet above jars, in addition to diffused daylight. Temperature average 20-22°C

(c) 3 days illumination as in b, remainder of first period and all of second period illumination by six 500 watt lamps in light chamber. Temperature average, 18-20°C

(d) First period in light chamber as in c, second period illumination as in b. The Cl content of the untreated cells was as follows

Experiment 1-101 milli-equivalents

" 2-103 "

" 3-108 "

TABLE A

Comparison of Conductivities of Normal Cell Sap and Sap from Cells Exposed to Solutions Containing KBr

(Phosphate buffer solutions with 5 milli-equivalents KBr)

Collection of cells.	Conditions of exposure	Concentration of Cl in cell sap.	Concentration of Br in cell sap.	Specific resistance of cell sap.	Percentage decrease in resistance.
		m. eq.	m. eq.	ohms	
B	6 hrs continuous illumination (1)	98.6	1.4	74.7	
B	6 days same conditions (1)	95.4	37.8	59.4	21
B	9 " " (1) (KBr 20 m. Eq)	97.0	53.1	56.2	25
C	No treatment.	105.5	0	77.2	
C	10 days (7 days intermittent and 3 days continuous illumination)	90.9	49.6	62.3	19
C	17 days daylight (3)	83.4	33.4	69.2	10
C	38 " continuous illumination (4)	69.1	64.8	58.9	24
D	No treatment.	101.1	0	74.3	
D	10 days (4 days daylight and 6 days continuous illumination) (5)	Not determined	64.5	51.2	31
D	9 days continuous illumination (6)	98.0	31.4	59.0	21
D	19 days daylight (7)	88.0	51.4	59.6	20
E	No treatment.	103.0	0	76.4	
E	16 days continuous illumination (8)	82.2	48.4	61.8	19
E	18 days continuous illumination (9)	80.0	56.6	54.8	28
E	20 days continuous illumination (10)	81.0	53.4	60.1	21
E	5 days continuous illumination (11)	105.0	41.0	54.9	28

(1) Continuous illumination by two 300 watt lamps suspended about 1 foot above jars. Temperature 22-26.5°C

(2) 7 days, diffused daylight, supplemented by two 300 watt lamps during periods of 7-8 hrs. 3 days light on at night also. Temperature 20-25°C

(3) Diffused daylight only. Temperature average approximately 20°C.

(4) Diffused daylight in addition to continuous illumination with two 300 watt lamps 2 feet above jars. Average temperature 20-22°C

(5) Diffused daylight and illumination during 6 nights by two 300 watt lamps 1 foot above jars

(6) Same conditions as in (4)

(7) Diffused daylight only. Room temperature.

(8) Same conditions as in (4)

(9) Illumination by six 500 watt lights light chamber. Average temperature approximately 20°C

(10) Same conditions as in (4)

(11) " " (9)

cell was constructed for use with slightly less than 1 cc of cell sap. Conductivity determinations were made in various experiments and certain typical data are given in Table X. In every case in which Br accumulated to any great extent a significant increase of conductivity occurred. These increases varied from 10 to 31 per cent, based on the values for sap from untreated cells. In certain instances, the increases of conductivity were of a magnitude very similar to those which would be obtained by adding to normal sap an equivalent amount of KBr, but in other cases, the increase is decidedly less than would correspond to this condition, as might be expected, considering that Cl ions have been displaced. Unpublished data indicate that an exchange of bases, especially sodium for potassium, may also occur under some circumstances. If these exchanges are taken into consideration, there is nothing in the conductivity data to suggest that an appreciable amount of Br has been organically combined. Other reasons for this view, previously discussed in connection with the chloride content of the sap also apply to the bromide content.

While the quantitative responses of different lots of cells collected at different times of the year are not exactly the same, there are one or two general relations which are strongly suggested by the available data. In several experiments, a large accumulation of Br took place with little or no loss of Cl. In these cases, the light and temperature conditions were very favorable to the accumulation and a high concentration of Br was attained in the cell sap in a relatively short period of time. In comparing two specific experiments conducted under different conditions, we note that in 6 days, with a high intensity of continuous illumination, 37.8 milli-equivalents of Br were accumulated with a loss of 3.2 milli-equivalents of Cl. In the other experiment, with diffused daylight alone, and with a lower temperature, 17 days were required to accumulate 33.4 milli-equivalents of Br and the loss of Cl was 22.1 milli-equivalents. While these relations can only be suggested at the present time, it is evident at least that a gain of Br and loss of Cl do not proceed necessarily at the same rate and that light, temperature, and time are important factors in all processes.

It cannot be proved, of course, that exchanges of ions are, strictly speaking, involved. Since it is also possible that cations may be

displaced, as well as anions, it could be assumed, for example, that KBr or K and Br ions entered the cell and that $NaCl$ or Na and Cl ions left the cell. However, the course of events is certainly more complex than this statement would imply.

There are various other data which can be discussed later when certain additional experiments have been made, but a few preliminary observations may be made now incidentally. With regard to the effects of hydrogen ion concentration on the accumulation of Br , our present results do not show that these effects are the same as those obtained in the studies on nitrate, which accumulates much more slowly than Br . The influence of certain other anions on the accumulation of Br is striking, and in single salt solutions, the nature of the cation plays a very important rôle.

DISCUSSION

In most of the investigations on the absorption of substances by plant cells, the latter are assigned a more or less passive rôle and the principal conclusions are concerned with explanations of alterations of permeability occurring under diverse conditions. This, however, can be only a partial view of the situation. From the point of view of the growth and nutrition of the plant, it is highly essential to emphasize the ability of the cell to concentrate or, in the sense of the word as recently employed by Osterhout, to accumulate substances in its interior. All the evidence now available shows that it is possible for certain inorganic elements to be taken out of a dilute solution and stored in a solution of much higher concentration inside the cell. It is true that such processes may take place relatively slowly, but, nevertheless, often at an appreciable rate, as we have shown with regard to Cl and Br . In most physiological experiments which have been reported, no adequate idea of the intake of inorganic elements by plant cells has been obtained because the time intervals employed were too short.

The ability of living cells to concentrate substances has, of course, been recognized by various writers and in this connection, it seems worth while to quote the following from a recent book by Lillie¹¹

¹¹ Lillie, R. S. *Protoplasmic action and nervous action*, University of Chicago Press Chicago 1923

"But since these compounds (crystalloidal compounds) do, in fact, gain entrance to the cell, at least at certain times, it is clear that the problem of cell permeability is not a simple one. Apparently, we must conclude that the entrance or exit of substances by simple diffusion is, in most cases, a different phenomenon from their entrance, or exit, under physiological conditions. The processes of absorption and secretion are, in fact, special activities, requiring the performance of work by the cell. The distinction between a passive or purely physical permeability and an active or physiological permeability thus seems to be a necessary one."

In view of the importance of such distinctions, it is regrettable that the term permeability has been used to describe so many types of phenomena. The general adoption of the term "accumulation" to designate certain processes, as in the recent usage of Osterhout, might serve to clarify discussion.

The experimental data presented in this article seem to offer definite evidence that light is an essential factor in the accumulation of Br or Cl by *Nitella* cells. Blackman and Paine, Trondle, Lepeschkin¹² and several others, have described the effects of light on permeability, but in these investigations, the methods were indirect and permeability, as such, was the chief point of interest. Results such as we have obtained cannot be explained by the statement that light simply increases cell permeability. Light obviously contributes energy to a system, and it would seem necessary to assume that this energy, which, under appropriate conditions can be stored, may be utilized to bring about a movement of solutes from a region of low concentration to one of higher concentration.

In our earlier work, which has been confirmed, we found that Cl did not diffuse out of uninjured cells into the various types of solution which were tried, and it was questioned whether such outward movement of Cl could take place in the absence of injury. It now seems certain that an exchange of Br for Cl may take place (or its equivalent), and present information would not point to injury as the primary cause of the exchange, which seems to occur to an appreciable extent only with elements which can be absorbed or accumulated by the cell with relative rapidity. Notwithstanding the existence of such exchange phenomena, Br and Cl can both accumulate in the cell in

¹² See review by Stiles, W., Permeability, Wheldon and Wesley, London, 1924

concentrations much higher than those present in the external solution, so that our views with regard to concentration gradients are not altered, but only extended.

It is now of interest to consider the suggestions recently advanced by Osterhout^{12, 14} to the effect that ions may not be able to penetrate living protoplasm, but that penetration is confined to undissociated molecules. We have been accustomed to assuming, in dealing with solutions of strong electrolytes such as those used in the experiments on *Nitella*, that the interpretation of the results could best be made in terms of ions. The dilute character of the solutions, existence of exchange phenomena apparently involving ions, the influence of one ion on the absorption of another, are some of the reasons which would seem to favor an interpretation in terms of ions, as far as the particular phenomena we have been investigating are concerned.

It is very possible the experiments carried out by Osterhout on certain chemical systems are not inconsistent with an hypothesis of ionic penetration as supplied to other systems. In his studies on the absorption of H_2S and CO_2 by *Valonia*, evidence was found that the equilibria between the sap and the sea water medium could be explained most logically on the basis that ions could not penetrate the cell. The question then arises whether the failure of the ions of the H_2S and CO_2 systems to penetrate necessarily implied that Br and Cl ions cannot do so. While the question is not now capable of a definite answer, several points of difference between the experiments with H_2S and CO_2 , and the experiments with Br and Cl should be noted. In the first place, the time periods involved are very much longer in the latter case, so that opportunity would be afforded for the very slow entrance of ions. In the case of the studies on H_2S , with *Valonia* it was not found that a higher concentration of sulfide was attained inside the cell than outside, while *Nitella* cells show a marked ability to concentrate Br or Cl in the sap. Then, in the H_2S and CO_2 systems, gaseous components were present, and it is quite possible that these were involved in such a way as to make it very uncertain whether any direct comparison can be made between the results

¹² Osterhout W. J. V. and Dorcas M. J. *J. Gen. Physiol.* 1925-26 ix, 255

¹⁴ Osterhout W. J. V. *J. Gen. Physiol.* 1925-26 viii, 131

obtained with Cl and Br, and those with CO_2 and H_2S . Still another point of difference is found in the temperature coefficient. Osterhout found a low coefficient for the entrance of H_2S , while in our experiments the coefficient for the accumulation of Br is of the order of magnitude of a chemical process.

Looking at the whole question from another point of view, it is not certain that we are now in a position to decide whether we are dealing with undissociated molecules or with ions, for the reason that intermediate chemical compounds may be formed, the chemical nature of which is unknown. If combinations of this sort are, in fact, involved, information concerning their character and the energy relations of their formation or dissociation would appear to be indispensable to an understanding of the mechanism of penetration of those substances which can accumulate in the cell sap of plants. It will also be necessary to determine whether these phenomena are concerned at all with electrical potential differences. In any case, the energy relations implied in the ability of living plant cells to concentrate substances require consideration irrespective of the mechanism of accumulation.

CONCLUSIONS

1 By the use of a special analytical technique it has been possible to study the accumulation of halogens in the cell sap of *Nitella*.

2 From a dilute solution, Br may be accumulated in the sap in a concentration much greater than that of the external solution. The conductivity of the sap may be markedly increased by such accumulation. The process is a slow one so that a month or more may be required to approach equilibrium.

3 Cl may be lost from the cell as a result of the accumulation of Br and *vice versa*. Other reciprocal relations between Cl and Br are indicated.

4 At equilibrium practically as much Br accumulated in the sap with an external solution containing 1 milli-equivalent of Br as with one containing 5 milli-equivalents.

5 Light energy was indispensable to the accumulation of Br. The temperature coefficient was characteristic of a chemical process.

THE EFFECT OF THE pH ON THE GERMICIDAL ACTION OF SOAPS

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That soaps possess a marked germicidal action has been known since the time of Robert Koch (1881) This valuable property finds every-day application in the sterilization of the skin dishes, and soiled clothing Soaps are furthermore of interest to the bacteriologist because they, or substances similar to them, very probably have something to do with the body's defence against infections (see Flexner's introduction to the paper of Lamar, 1911, a)

Solutions of soaps having 12 or more carbon atoms in the molecule are alkaline in reaction, because of hydrolysis The longer the chain of carbon atoms, the greater is the hydrolysis and the more alkaline is the solution Most investigators of the germicidal action of soaps are agreed that this alkaline reaction favors the destructive effect on the bacteria Reichenbach (1908), working with *B. coli* as test organism, found that the more strongly hydrolyzed and therefore more alkaline soaps were most germicidal addition of alkali increased the germicidal titer Lamar (1911, b) found sodium oleate to be more hemolytic than oleic acid, this difference he ascribed to the lower solubility of the acid Nichols (1919-20) found that the soaps commonly used in washing dishes (the resinates, stearates, and palmitates) were precipitated and lost their germicidal action when the reaction was brought to pH 7.0 or more acid Further than this, there seem to be no observations recorded in the literature on the effect of the pH on the germicidal action of the soaps

EXPERIMENTAL.

Soaps of the following normal fatty acids were investigated butyric, caproic, caprylic, capric, undecylic, lauric, tridecylic, myristic,

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pentadecylic, palmitic, stearic, oleic, and ricinoleic. The butyric, caproic, and oleic acids were Kahlbaum's, the remainder were obtained from the Eastman Kodak Company. The soaps were prepared by adding the theoretical quantity of fatty acid to N/5 KOH. Potassium soaps were chosen because of their greater solubility. Walker (1924) has shown that the sodium and potassium soaps have prac-

TABLE I
Composition of Buffer Mixtures

pH	N/5 KH_2PO_4	N/5 KOH	N/10 glycine	N/10 KOH
	cc	cc	cc	cc
5.0	9.90	0.10	10.0	0
5.5	9.71	0.29	10.0	0
6.0	8.97	1.03	10.0	0
6.5	7.67	2.33	10.0	0
7.0	6.28	3.72	10.0	0
7.5	5.50	4.50	10.0	0
8.0	5.17	4.83	9.75	0.25
8.5	5.10	4.90	9.0	1.0
9.0	5.10	4.90	8.0	2.0
9.5	5.10	4.90	6.5	3.5
10.0	5.10	4.90	5.5	4.5

TABLE II
Composition of Lactic Acid-Potassium Lactate Buffer Mixtures

pH	N/10 potassium lactate	N/1 lactic acid
	cc	cc
5.0	10.0	0.0625
4.7	10.0	0.125
4.4	10.0	0.25
4.1	10.0	0.50
3.8	10.0	1.00

tically the same germicidal value. Before each experiment, serial dilutions of the soaps required were made with sterile distilled water.

The pH of the soap solutions was controlled by the addition of buffer mixtures, whose composition is given in Tables I and II.

The composition of the phosphate and glycine buffers was calculated from the data given in Clark (1922). The pH of the glycine-

KOH mixtures is for 37°C. The phosphate mixtures were made up in larger quantities, the glycine-KOH mixtures were made up fresh for each experiment and added to the phosphate.

Five different species of test organisms were used. These were

1 *Streptococcus pyogenes*. The strain used was the "H" strain first described by Gay and Stone (1920), its extremely high virulence for rabbits has been maintained by constant animal passage. This strain is hemolytic, it grows vigorously and diffusely in infusion broth.

2 *B. diphtheriae*. The "Park Williams No. 8" was the strain used.

3 *Staphylococcus aureus*. An old laboratory stock culture was employed.

4 *B. typhosus*. The "Pfeiffer" strain was used.

5 *Vibrio cholerae*. The strain used was an old stock culture of unknown origin.

The germicidal tests were performed in the following manner:

The required buffer solutions were sterilized, when cool, they were inoculated with the test organisms and well mixed. Then 0.5 cc. quantities were pipetted into series of small sterile test-tubes. The inocula were such that each 0.5 cc. of the buffer fluid contained 0.04 cc. of an 18 to 24 hour broth culture of *Streptococcus pyogenes*, *B. diphtheriae*, or *V. cholerae*, or 0.02 cc. of *Staphylococcus aureus* or *B. typhosus*. Then 0.5 cc. quantities of the soap solutions (serial dilutions of which were prepared in advance) were added to each tube. Finally each tube was gently rotated while in a slanting position and placed in the water bath at 37°C. At the end of 30 minutes, 2 hours, and 18 hours, a 4 mm. loopful from each tube was subcultured on plates of blood agar (*Streptococcus*, *B. diphtheriae*, and *V. cholerae*) or plain agar (*Staphylococcus* and *B. typhosus*). In case the tube contained a precipitate of insoluble fatty acid, the precipitate was well stirred with the platinum loop on subculture. The plates were incubated at 37°C. for 48 hours and read. In several experiments duplicate subcultures were made: the first set on agar plates, the second set in tubes of infusion broth. No essential differences were observed.

The soap solutions added were in certain instances strongly alkali

line The question thus arises, how adequate are these buffer mixtures to resist change of pH on the addition of this alkali? The alkaline end of the series—from pH 8.0 on—is very well buffered against weak alkalis such as the soaps, the acid end, on the other hand, is but poorly buffered against any but very dilute solutions. It was found necessary to test the final pH of the solutions with indicators to determine where any serious shifting of pH had occurred. The less hydrolyzed soaps gave very little trouble. Those with fewer than 10 carbon atoms could easily be adjusted with acid before being added to the buffer mixtures. The potassium laurate, in concentrations

TABLE III

	Acid limit of tolerance		Alkaline limit of tolerance	
	2 hrs	18 hrs	2 hrs	18 hrs
	pH	pH	pH	pH
<i>Streptococcus</i>	5.5	6.0	9.5	9.0
<i>B. dysenteriae</i>	4.4	4.7	10.5	10.0
<i>V. cholerae</i>	5.5	6.0	9.0	8.5
<i>Staphylococcus</i>	3.8	4.4	10.5	10.0
<i>B. typhosus</i>	4.4	5.0	10.0	9.0

The pH values given indicate the most acid or alkaline tube that gave growth on subculture. The tests were conducted in the same manner as the germicidal tests with soap, except that distilled water instead of soap solution was added to the buffer fluids.

of N/160 or less, caused no serious shifting of pH except in the most acid of the phosphate mixtures. The same was true of the soaps of higher molecular weight when the concentration was N/320 or less. When any shift in the pH of a significant tube occurred, the observed pH (colorimetric) was recorded. As a matter of fact, corrections of this nature were not often required.

Inasmuch as an excess of either acid or alkali is destructive to bacteria, repeated tests were made to determine the acid and alkaline limits of pH tolerated by the five organisms here studied. The results are given in Table III.

The results of the germicidal experiments with soaps are given in Figs. 1 to 7. Attention must be called to the fact that the ger-

micidal titers given in these figures refer, not to the amount of soap or fatty acid in solution, but to the total amount added irrespective of whether a precipitate formed or not. Thus, when N/10 potassium stearate was brought to pH 6.0, practically all of it was precipitated out. When such a mixture was found to be without germicidal properties, it was recorded as having a titer of <N/10. Again, when N/640 potassium myristate was brought to pH 6.0 (as in Fig. 1) a precipitate formed, and the actual concentration of myristate in solution became much less, yet it was recorded as N/640. This procedure was adopted for two reasons: first the difficulty of estimating the amount of fatty acid actually in solution, and second, the fact that the substances in solution are in equilibrium with the precipitate, so that the latter cannot be regarded as wholly inert.

A study of the experimental data brings out the following facts:

- 1 Potassium butyrate is non-germicidal in a concentration of N/10 at all pH values within the limits given in Table III.

- 2 The lower members of the saturated series of soaps are most germicidal in an acid reaction. The most striking example of this was found in the case of potassium caprate and *Staphylococcus*; the titer here was 1000 times as great at pH 4.4 to 4.7 as at pH 9.0 to 10.0.

- 3 The curves for the higher members of the saturated series are the reverse of those for the lower members, showing greater germicidal action when the pH is alkaline. Certain soaps (such as the myristate and the palmitate with *Streptococcus*) are intermediate in their behavior, showing a definite minimum in their activity near the neutral point.

- 4 With increasing molecular weight of the soap, the germicidal titer increases to a maximum and then diminishes. The point at which this inflection occurs varies with the pH and the organisms (Figs. 5 and 6). Thus, with *B. typhosus* at pH 5.5 the titer rises from caproic to capric acid, and then falls off very rapidly through undecylic acid to lauric acid, where no germicidal action could be demonstrated. With the other four organisms tested, the maximum for the acid range was reached at lauric and tridecyl acids, with a somewhat slower falling off of the titer with increasing molecular weight. In the alkaline pH range, the germicidal action toward all five organ-

isms increases with the molecular weight to the palmitate and then diminishes

5 The oleate and ricinoleate were not tested against all five organisms. Fig 7 shows that the oleate with *Streptococcus* and the ricinoleate with *B. dysenteriae* are far more effective in acid than in neutral or alkaline ranges. The oleate with *B. dysenteriae* gave a curve quite unlike any other that was obtained, with maximal germicidal action at pH 6.5 and 7.0. The oleate showed no action on *Staphylococcus* and very little on *B. typhosus*, and then only at the most alkaline reactions. This confirms the observations of Reichenbach (1908) and Walker (1924).

From this data it is clear that the soaps have a germicidal power that varies with their structure and the species of germ acted upon, and that the corresponding fatty acid may likewise be germicidal, often far more than the soap.

The question arises, what is the cause of the sudden drop in the curves (Figs 5 and 6) after a maximum has been reached? Why are the curves in Figs 1 to 4 reversed as we pass from soaps of lower to higher molecular weight?

One reason for this is the diminishing solubility of the soap or fatty acid as the molecular weight increases. Thus, the most concentrated solution of potassium laurate that remains clear at pH 6.0 is N/5120. More concentrated solutions contain insoluble matter in suspension. It is impossible to determine whether N/160 dissolved laurate or palmitate would kill the typhoid bacillus at pH 6.0, because that amount cannot be obtained in solution.

There is also evidence to show that the germicidal power of the fatty acids also actually diminishes with increasing molecular weight, after a maximum has been reached. It is suggested that this may be due to diminished solubility of the germicide in the bacterial protoplasm. In the case of *B. typhosus* (Fig 6), the sudden drop from capric to undecylic acid can be explained only by a diminution in germicidal power.

A comparison of the germicidal titers for the three different time intervals gave some rather interesting results. The following combinations ran practically the same titers for the 30 minute, 2 hour, and 18 hour periods. *Staphylococcus* with soaps of 12 or fewer carbon

atoms, *B. typhosus*, *B. diphtheriae*, and *V. cholerae* with 11 or fewer carbon atoms. With soaps of higher molecular weight, the titers were increased by lengthening the incubation period. This increase, however, was not always uniform over the whole pH range. Thus, for the 18 hour period, the curve for the myristate and *Staphylococcus* (Fig. 1) was flattened out without being raised at the ends, while the palmitate and stearate curves were shifted to the left without any change in their shape. On the other hand, the myristate curve for *V. cholerae* (Fig. 3) instead of being flattened, was strongly raised at both ends when the incubation period was increased to 18 hours.

The tests described were carried out in a salt concentration of N/20. A detailed study of the effect of salt on the germicidal action of soaps

TABLE IV

The Effect of the Salt Concentration on the Germicidal Titer of Potassium Laurate on B. diphtheriae

Salt concentration. (Potassium phosphate.)	Germicidal titers.		
	pH 6.0	pH 7.0	pH 8.0
N/10	N/10 240	N/1 280	N/320
N/25	N/5 170	N/640	N/160
N/100	N/2 560	N/320	N/160

Temperature 37°C. Time of incubation 2 hours.

was not undertaken, but a few experiments were made which indicated that the addition of salts increases the germicidal action. One such experiment is shown in Table IV.

This effect of salt upon the germicidal action of soaps and fatty acids is similar to the effect of salt upon phenol, another organic acid. In the latter case, the effect of salt was explained by Spiro and Bruns (1898) in this manner: When bacteria are added to a solution containing phenol, the equilibrium concentrations of $\frac{\text{phenol in bacteria}}{\text{phenol in water}}$ will depend upon the relative solubility of the phenol in the two phases. If anything is added to the water phase (such as alcohol) that increases the solubility of the phenol in it, then the concentration of phenol in the bacteria is diminished and likewise germicidal action. Con

This would affect the distribution coefficient of the germicide between germs and test fluid. We do not know what changes occur in the pH of living bacterial protoplasm when the pH of the outside fluid is altered. However, Coulter (1924-25) has shown that when acid is added to a suspension of erythrocytes, the cell contents do not become as acid as the outside fluid. If this is the case with bacteria, an acid outside pH would be accompanied by a much less acid pH of the bacterial protoplasm. As acids diminish the solubility of soaps, an acid reaction of the outside fluid should, therefore, increase the concentration of soap (or fatty acid) in the bacteria. If, however, the added acid precipitates most of the soap out of solution, the actual concentration within the bacteria would be diminished, even though the relative concentration is still greater than that in solution in the outside fluid.

4. The greater germicidal activity of the fatty acid may be due to the fact that the acid is less dissociated than the soap. There is a good deal of evidence to show that undissociated molecules penetrate more readily into protoplasm than do ions (Osterhout, 1925-26). Michaelis and Dernby (1922) believe that the germicidal effect of the organic bases studied by them is due entirely to the undissociated molecules; they show that the curve for the germicidal titer closely parallels the dissociation residue curve for a weak base. Scudder (1914) gives the value of K_a for caprylic acid as 1.44×10^{-4} ; the dissociation residue for this acid corresponds very closely with the germicidal titer curves for the same acid (Figs. 1 to 4). The latter, however, are logarithmic curves, hence it would appear that the logarithm of the germicidal titer varies with the dissociation residue of this acid. This means either that other variables enter into the germicidal effect or that the dissociation residue affects the germicidal titer in two or more different ways (*e.g.* by affecting the surface tension or the distribution coefficient as well as the ability to penetrate).

SUMMARY

1. The effect of the pH on the germicidal action of soaps has been studied. The lower members of the series were found to be most active in acid solution, the higher members, in alkaline. The point of transition varied with the test organism used.

2 This is probably due to the effect of the pH on the dissociation residue and on the solubility of the soap. The dissociation residue may affect the germicidal titer by modifying the surface tension, the penetration into the bacteria, and the partition coefficient of the germicide between bacteria and water.

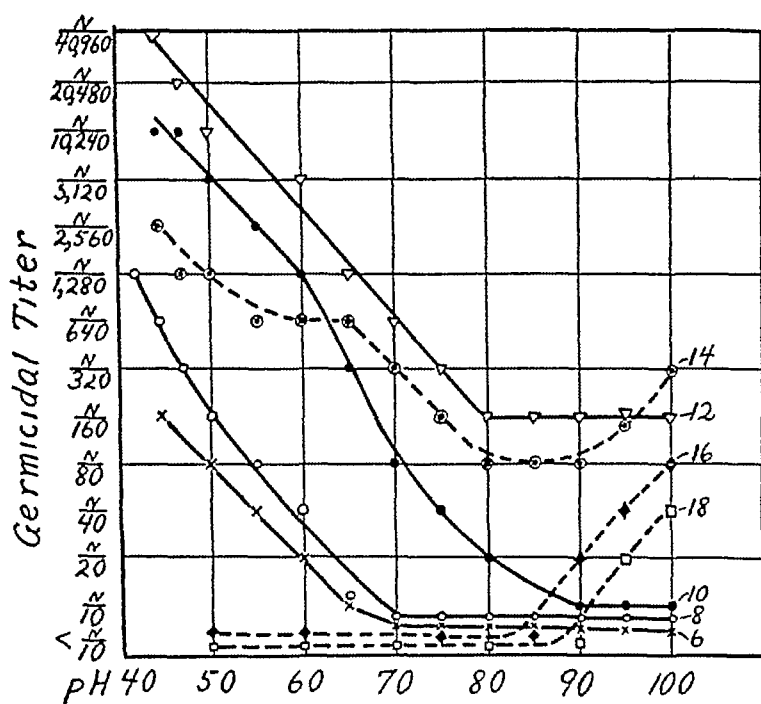


FIG 1 Germicidal titers of saturated soaps for *Staphylococcus aureus*. The incubation period was 2 hours at 37°C. The numbers at the right of the curves designate the carbon atoms in the molecule. The curves for the undecylate, tridecylate, and pentadecylate are omitted from the figure for simplification, they would occupy an intermediate position between the adjacent soaps with an even number of carbon atoms.

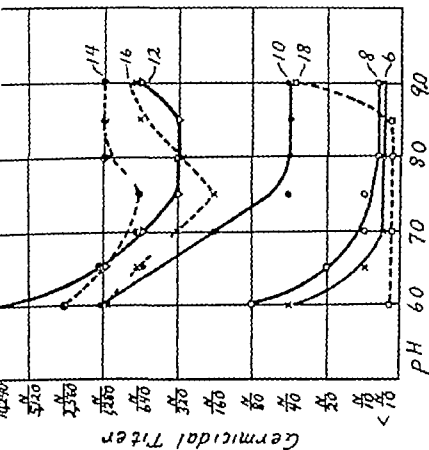


FIG. 2.

FIG. 2 Germicidal titers of saturated soaps for *Streptococcus pyogenes*. The incubation period was 2 hours at 37°C. The numbers at the right of the curves designate the carbon atoms in the molecule. The curves for the undecylate tridecylate and pentadecylate are omitted; they would occupy an intermediate position between the adjacent soaps with an even number of carbon atoms.

FIG. 3 Germicidal titers of saturated soaps for *V. cholerae*. The incubation period was 2 hours at 37°C. The numbers at the right of the curves designate the carbon atoms in the molecule. The curves for the undecylate tridecylate and pentadecylate are omitted; they would occupy an intermediate position between the adjacent soaps with an even number of carbon atoms.

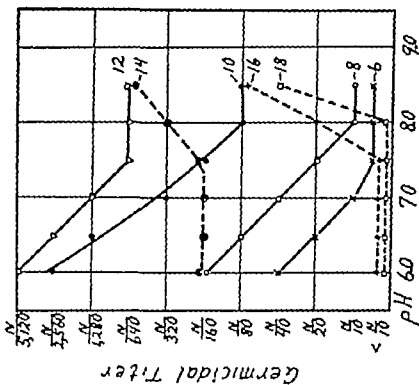


FIG. 3

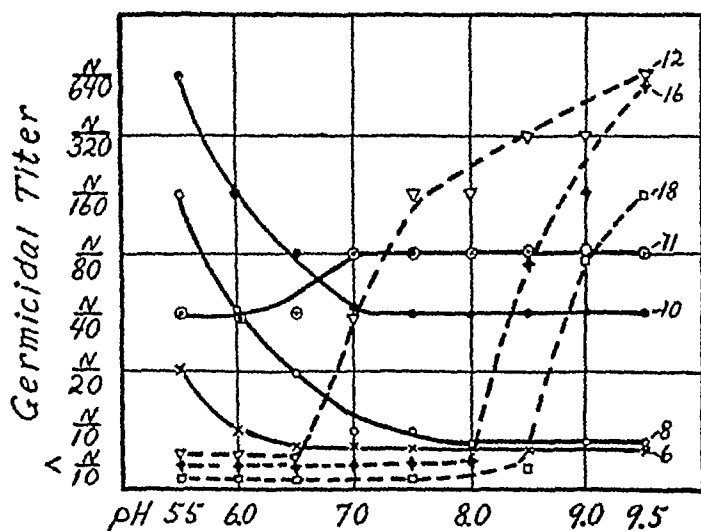


FIG 4 Germicidal titers of saturated soaps for *B typhosus* The incubation period was 2 hours at 37°C The numbers at the right of the curves designate the carbon atoms in the molecule The curves for the soaps with 13, 14, and 15 carbon atoms were omitted to simplify the figure

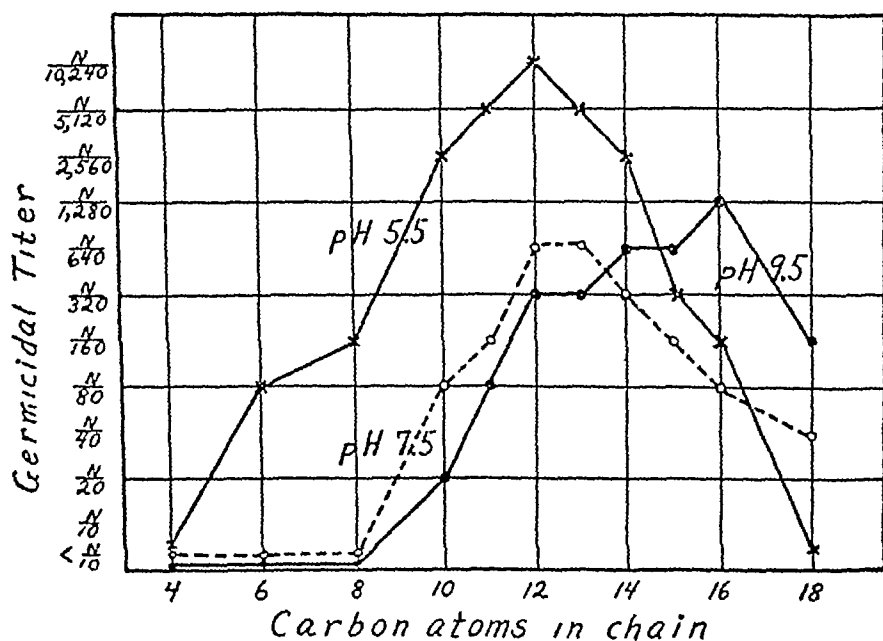


FIG 5 The germicidal titers of saturated soaps for *B diphtheriae* The incubation period was 2 hours at 37°C The soaps are designated by the number of carbon atoms in their molecule

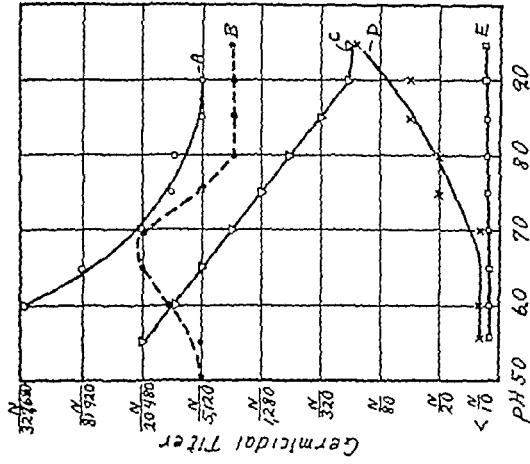


FIG 7

FIG 6. The germicidal titers of saturated soaps for *B. typhosus*. The incubation period was 2 hours at 37°C. The soaps are designated by the number of carbon atoms in their molecule.

FIG 7. The germicidal titers of two unsaturated soaps. The incubation period was 2 hours at 37°C. A Potassium oleate and *Streptococcus pyogenes* B Potassium oleate and *B. diphtheriae* C Potassium ricinoleate and *B. diphtheriae*. D Potassium oleate and *B. typhosus* E Potassium oleate and *Staphylococcus aureus*

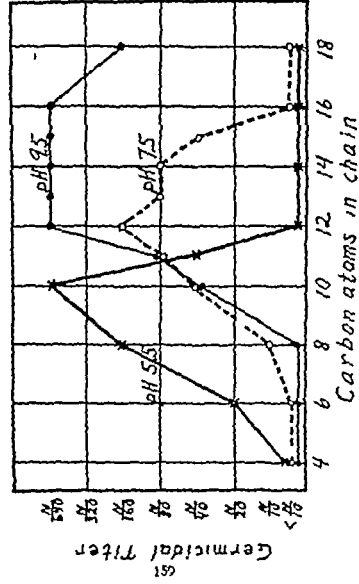


FIG 6

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THE SWELLING PRESSURE OF GELATIN AND THE MECHANISM OF SWELLING IN WATER AND NEUTRAL SALT SOLUTIONS

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The swelling of gelatin when placed in aqueous solution may be readily separated into three types. The swelling in acid or in alkali and the effect of neutral salts on this swelling have been shown by the work of Procter and Wilson,¹ and Loeb² to be due to the osmotic pressure of the ions of the electrolyte, in accordance with the Donnan equilibrium. The initial swelling of dry gelatin in water—which evidently is not connected with the Donnan equilibrium—has been carefully studied by Katz,³ who was able to show that the heat effects, volume, pressure, and vapor pressure changes were strictly analogous to those observed in the formation of concentrated solutions of many substances, and that the system as a whole behaved as an ideal concentrated solution. The large amount of heat liberated indicates strongly that a reaction occurs between the water and gelatin, resulting, presumably, in the formation of a gelatin hydrate. When sufficient water has been added, however, to reduce the gelatin concentration to less than 50 per cent, the heat effects become very small and yet the gelatin may swell, under favorable conditions, until the concentration of gelatin is 5 per cent or less. It appears improbable that this water is all combined with the gelatin in the form of a hydrate, and it seems necessary to consider this as a third distinct type. It is this type of swelling which is discussed in the present paper.

The influence of salts on this tertiary swelling was found by the

¹ Cf. Wilson J. A., in Bogue R. H., *The theory and application of colloidal behavior*. New York and London 1924, 1, 1.

² Loeb J. *Proteins and the theory of colloidal behavior*, New York and London 1922.

³ Katz J. R. *Kolloidchem. Beihefte* 1917-18 ix 1.

writers⁴ to be closely parallel to the effect of salts on the osmotic pressure of gelatin solutions, and it was further shown that these effects could not be accounted for on the basis of a Donnan equilibrium. It was suggested that this swelling was a purely osmotic phenomenon and that the effect of salts was due primarily to their influence on the osmotic pressure. It is evident that the problem would be greatly simplified if the pressure with which the water was drawn into the solid gelatin could be measured instead of merely the rate or extent of swelling. This "swelling pressure," although analogous to osmotic pressure of a gelatin solution, cannot be measured in the same way, since if solid gelatin is enclosed in a rigid membrane the pressure will be exerted on the walls of the membrane. It was found possible however, by placing the gelatin outside a porcelain thimble coated with collodion, to measure this swelling pressure. The measurements show that the pressure measured in this way is but little less than the osmotic pressure of the same gelatin when liquid, and they corroborate the idea that the swelling in water and neutral salt solutions is due to the osmotic pressure of the solution held in the meshes of the gel.

Experimental Method

Gelatin —Isoelectric gelatin was prepared as described by Loeb² and all measurements were made at pH 4.7.

The method of making the measurements is shown in Fig. 1. The Chamberland filter was coated with collodion by pouring collodion slowly on the surface while the thimble was rotated mechanically. The tube was partially filled with gelatin of the desired concentration and the thimble and manometer tube, previously filled with water, inserted as shown in the figure. The tube was then placed in a water bath at the desired temperature and the pressure measured after equilibrium was reached. A number of experiments were made to determine whether the thickness of the gelatin layer or the previous treatment of the system had any influence on the final reading. The results of some of these experiments are shown in Table I. The equilibrium pressure is evidently independent of the thickness of the gelatin layer, and the temperature effects are reversible. It was also found that the final pressure was independent of the initial pressure, *i.e.*, it is a true equilibrium value. The results, when the tube was completely filled with gelatin were much more regular, and this method was used in most of the experiments. The figures are the averages of 4 to 8 measurements and are reliable to about 5

⁴ Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925-26, viii, 317.

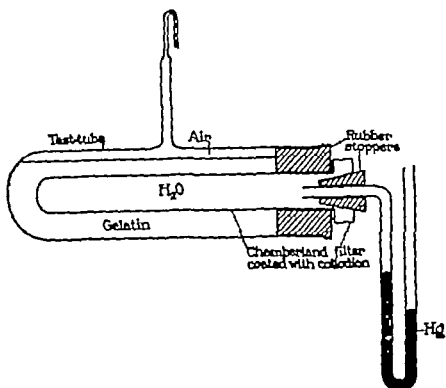


FIG. 1 Apparatus for measuring swelling pressure of gelatin

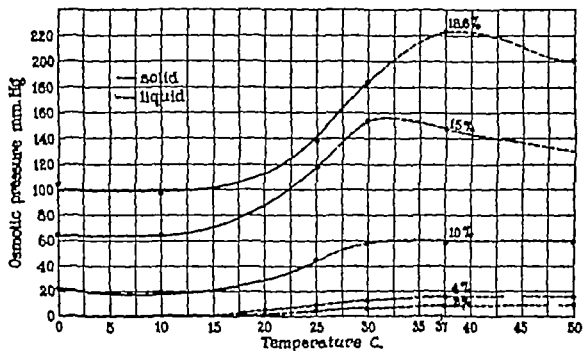


FIG. 2 Osmotic or swelling pressure of various concentrations of gelatin at different temperatures

per cent In working with the thin coatings of gelatin it was occasionally noticed that the water evaporated from the gelatin and condensed on the surface of the tube even when the manometer showed high negative pressure on the water in the thimble This result is exceedingly difficult to account for except on the basis of some temperature difference, and is perhaps similar to von Schroeder's⁵ anomalous findings The pressure of the gelatin when liquid was determined in a rocking osmometer as previously described⁴

Effect of Temperature and Concentration of Gelatin on the Swelling Pressure of Gelatin

The result of a series of experiments in which the pressure of various concentrations of gelatin as measured over a range of temperatures

TABLE I

Effect of Thickness of Gelatin Layer and of Previous Treatment on Swelling Pressure of 10 Per Cent Gelatin at 25°C

Gelatin on thimble	Other conditions	Pressure
"		mm.Hg
25	Suspended in closed tube in air	49
48	" " " " " "	54
195	" " " " " "	44
250	Tube filled with gelatin	45
250	At 0°C for 10 min before placing at 25°C	48
250	" 0° " " 18 hrs " " " 25° "	46
250	" 37° " " " " " " " 25° "	47

is shown in Fig 2 The solid line indicates that the gelatin was solid and the dotted line indicates liquid gelatin It will be seen that in high concentrations of gelatin the pressure increases with decreasing temperature to give a maximum near 37°C, and then decreases quite sharply, as the gelatin solidifies, to become nearly independent of the temperature below 15°C The negative temperature coefficient in the high concentrations is due presumably to the large positive heat of solution⁶ and so does not occur in the low concentrations The drop

⁵ von Schroeder, P, *Z physik Chem*, 1903, xlv, 109

⁶ Cf Findlay, A, *Osmotic pressure*, London and New York, 2nd edition, 1919,

in the pressure between 30° and 20°C occurs also in the low concentrations where the gelatin does not solidify, and in these cases approaches zero. In these dilute solutions a precipitate forms and hence no osmotic pressure would be expected. If gelatin is considered as a single chemical substance, these results are evidently exceedingly difficult to understand. The drop in the pressure below 35°C would result if an insoluble substance is separating out, but if the solution contained only this substance the pressure must become independent of the initial concentration as soon as the solid phase appears. Thus

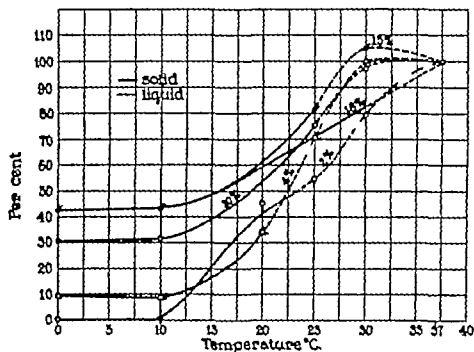


FIG. 3. Osmotic or swelling pressure of gelatin of different concentrations as per cent of value at 37°C.

is not the case. The experiment is analogous to Sørensen's⁷ results on the solubility of the globulins, in that the osmotic pressure (concentration) increases with the total amount of substance added even in the presence of the solid phase. This is the expected result if it is considered that gelatin is a mixture of (at least) two substances, one of which is easily soluble over the range of temperature studied, and the other of which is readily soluble above 35°C, but much less soluble below 15°C. The drop in the pressure between 35° and 15°C is due

⁷ Sørensen, S. P. L. *Proteins*. The Fleischmann Laboratories, New York, 1925.

then to the separation of the insoluble fraction. If the concentration is sufficiently high a gel results, if not, particles are formed containing the soluble material and there is no osmotic pressure of the solution as a whole. The osmotic pressure as per cent of the value at 37°C is plotted in Fig 3 and as a function of the concentration of gelatin in Fig 4. The percentage drop in the osmotic pressure is nearly constant in this range except for the very low concentrations. It will be noticed that the osmotic pressure increases more rapidly than the concentration, a result which can be predicted from the viscosity

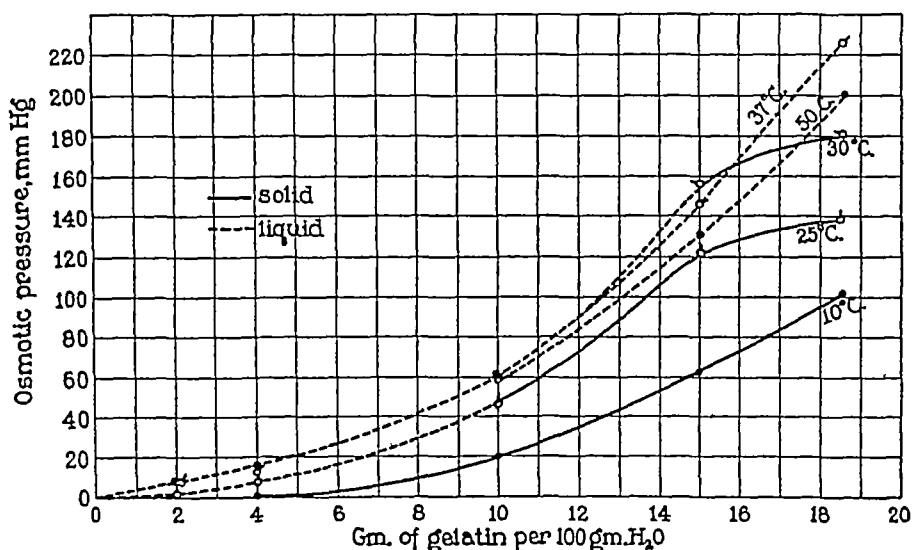


FIG 4 Concentration and swelling or osmotic pressure of gelatin at different temperatures

curves (Kunitz⁸) and which is due to the fact that a considerable quantity of the water is contained in the particles. (This effect will be discussed quantitatively in a subsequent paper.) The pressure also increases with the total concentration even when the gelatin is solid. This shows, as stated above, that the system cannot be considered as a saturated solution of one substance.

It should be possible, according to these results, to separate gelatin into two fractions: a soluble one, having high osmotic pressure, low

⁸ Kunitz, M., *J Gen Physiol*, 1925-26, ix, 715

viscosity, and no tendency to gel formation, and an insoluble one, having a limited solubility at low temperatures, a low osmotic pressure, and undergoing little or no swelling. It might also be expected, since the soluble fraction must be held in the meshes of the insoluble one, that isolation of the former would be much more easily accomplished than the separation of the insoluble from its accompanying soluble component. It was found possible after a number of attempts to separate gelatin into two fractions having nearly the properties described above. This was done by combining the temperature effect on the solubility with alcohol precipitation. The fractionation was carried out as follows:

Preparation of Insoluble Fraction

10 liters of 5 per cent isoelectric gelatin at 35°C. and 7.5 liters of 95 per cent alcohol added, 18 hours at 20°C. filtered with folded paper. Filtrate = No. 1

Precipitate. Made up to 8 liters with water, heated to 35°C., and 2 liters of alcohol added. Cooled to 20°C. and filtered. Filtrate rejected.

Precipitate. Made up to 8 liters, etc. as above, and repeated 4 times.

Precipitate. Made up to 2 liters with water, heated to 35°C. and 1.6 liters of alcohol added. Cooled to 30°C. Jelly like residue settled. Supernatant liquid decanted and rejected.

Residue. Made up to 2 liters, etc. as above, and repeated 5 times.

Final residue. Made up to 2 liters with water at 35°C., 800 cc. alcohol added and cooled to 10°C. Precipitate filtered and dried with alcohol and ether. 42 gm. marked insoluble fraction.

Preparation of Soluble Fraction

Filtrate No. 1. 24 hours at 0°C. and filtered at 0°C. Filtrate rejected.

Precipitate. Made up to 400 cc. with water, heated to 30°C. 400 cc. of alcohol added and cooled to 20°C. Filtered. Precipitate rejected.

Filtrate. 18 hours at 0°C. Filtered at 0°C. Filtrate rejected.

Precipitate. Made up to 200 cc. heated to 30°C., and 200 cc. of alcohol added. No precipitate on cooling to 20°C. Cooled to 0°C. 18 hours. Filtered at 0°C. Precipitate dried with alcohol and ether. 25 gm. marked "soluble fraction."

No evidence is at hand to show that either of these fractions is a chemical individual and on the contrary there is reason to suppose that the insoluble fraction still contains a considerable quantity of the soluble since it still swells, and further purification caused a still further

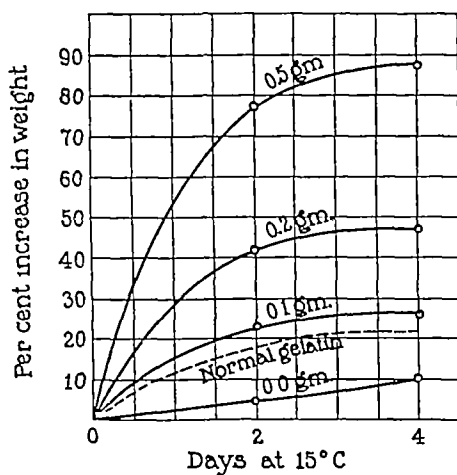


FIG 5 Effect of increasing amounts of soluble fraction on swelling of insoluble fraction 5 cc H_2O , 0.4 gm insoluble + noted amounts of soluble fraction at $15^\circ C$

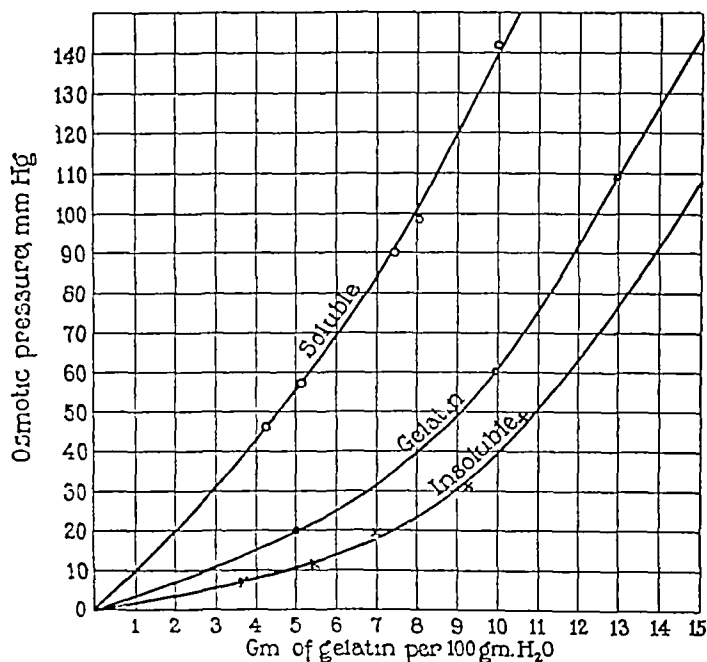


FIG 6 Osmotic pressure of various concentrations of gelatin and of the soluble and insoluble fractions

decrease in the osmotic pressure. Owing to the difficulty of obtaining sufficient material with which to work, however, the process was stopped at this stage.

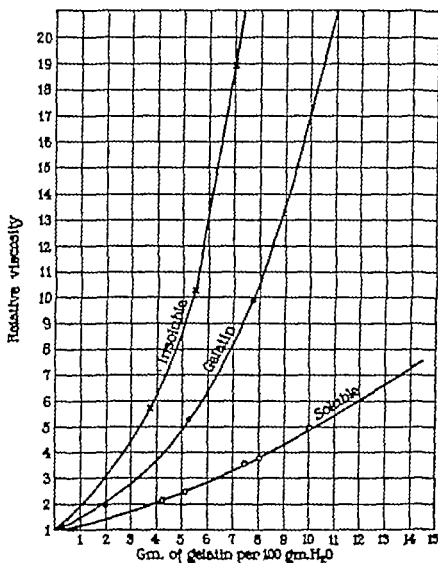


FIG. 7. Viscosity of various concentrations of gelatin and of the soluble and insoluble fractions.

Comparison of the Properties of the Two Fractions and of Gelatin

In accordance with the mechanism of swelling outlined above it would be expected that the insoluble fraction should swell very little while the soluble fraction should dissolve. Mixtures of the two should swell the more the greater the percentage of soluble material. Fig. 5 shows that this is actually the case. The swelling of the in

soluble fraction itself is probably due to the fact that it still contains an appreciable quantity of the soluble material, although it could of course be assumed that a different mechanism was responsible for this swelling

Figs 6 and 7 show the results of osmotic pressure and viscosity measurements. The viscosity of the insoluble fraction is much greater than that of gelatin while the osmotic pressure is less. The osmotic pressure of the soluble fraction is greater and the viscosity less, as would be expected. Since the insoluble fraction swells less than gelatin, it might be expected that the viscosity would be less instead of

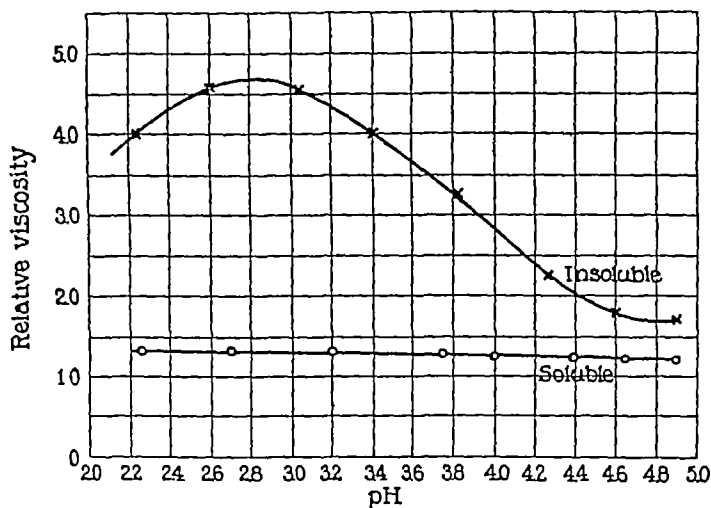


FIG 8 Effect of addition of HCl on the viscosity of 1 per cent solutions of soluble and insoluble fractions at 37°C

greater. It must be remembered, however, that although the individual particles swell less, the number of particles per gm is greater in the insoluble fraction than in gelatin, since the latter also contains the soluble material. Since the viscosity, according to Einstein, depends on the total volume occupied by the particles, the higher viscosity of the insoluble fraction is due to the fact that the increase in the number of particles capable of swelling more than makes up for the difference in the swelling of the individual particles. As was stated above, the form of the osmotic pressure curves can be quantitatively accounted for by the absorption of water in the particles. Qualitatively it can

be predicted that the substance having the highest viscosity should have the greatest curvature in the osmotic pressure-concentration curves and, as the figures show, this is the case. The marked difference in the viscosity of the soluble and insoluble fractions was ascribed above to the fact that the insoluble fraction in solution consisted largely of particles capable of swelling, while the soluble fraction did not. If this is the case, it would be expected that the insoluble fraction should show a marked pH viscosity effect, while the soluble

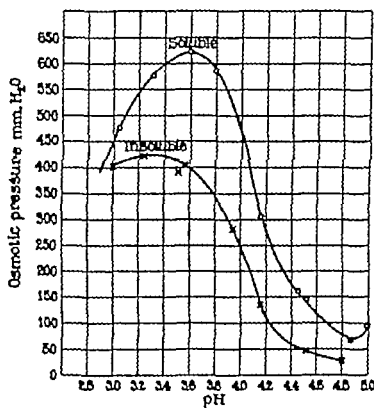


FIG. 9. Effect of addition of HCl on osmotic pressure of 1 per cent solutions of soluble and insoluble fractions at 37°C.

fraction should show little or none, since, according to Loeb,² the increase in viscosity on addition of acid is due to the swelling of such particles by the Donnan equilibrium set up in them. Fig. 8 shows that this is the experimental result. On the other hand, it would be expected that the effect of acid on the osmotic pressure would be about the same, since in this case the collodion sac is the particle and is evidently present in both cases. This expectation is also fulfilled as is shown in Fig. 9.

The titration curves of the two fractions, and the speed with which they are digested by pepsin, do not differ significantly from those of ordinary gelatin

The Effect of Temperature and Salts on the Osmotic Pressure

It was suggested above that the increase of pressure occurring when gelatin solutions or gels are raised from 25° to 37°C was due to the solution of the substance forming the network and corresponding to the insoluble fraction. It would be expected therefore that the temperature would have a still greater effect on the osmotic pressure of the insoluble fraction and a smaller effect on the soluble fraction. This

TABLE II

Effect of Temperature on Osmotic Pressure of Soluble and Insoluble Fractions 5 Per Cent Solutions

Temperature	Pressure in mm Hg	
	Soluble fraction	Insoluble fraction.
°C		
37	50 liquid	10 liquid
25	48 "	6 7 solid
20		5 3

is the result, as shown in Table II. The insoluble fraction still exerts pressure below 25°C which indicates, as stated above, that it still contains some soluble material.

As is well known, strong solutions of some neutral salts liquefy gelatin and, as was shown in a preceding paper,⁴ also increase the osmotic pressure. The action of these salts on gelatin at 15°C, and the effect of raising the temperature to 37°C are therefore similar except that the increase of osmotic pressure due to the salt is greater than that due to increasing the temperature. A solution of gelatin at 37°C contains particles capable of swelling, as is shown by the pH-viscosity effect. The osmotic pressure of such a solution could be increased in two ways.

First, the salt might increase the swelling of the particles. Since

in this process water is removed, while the number of particles remains the same, the result is an increase in the mol fraction, and a resulting increase in the osmotic pressure. Second, the salt might cause these particles to break up into smaller ones, thereby increasing the number of particles and hence the osmotic pressure. On the basis of the first assumption it would be expected that the salt should also increase the viscosity and further that the effect of temperature, in the presence of salt, would not differ very much from that in the absence of salt. On

TABLE III

Effect of Salts on Viscosity and Osmotic Pressure of 10 Per Cent Gelatin

Concentration of salt.	0	2 M NaSCN
Relative viscosity $\frac{\eta}{\eta_{\text{salt}}}$, at 37°C.	19.3	18.10
Osmotic pressure, mm Hg at 37°C	60	122
" " " " 25°C	44	113
" " " " 15°C	20	108
" " " " 3°C	20	102

TABLE IV

Effect of NaSCN on the Osmotic Pressure of 5 Per Cent Soluble and Insoluble Fractions of Gelatin at 37°C

Fraction.	Soluble.		Insoluble.	
	0	2M	0	2M
Concentration of NaSCN inside and outside	50	61	8	35
Osmotic pressure, mm Hg				

the second assumption there would be expected no change or a decrease in the viscosity and the temperature effect on the osmotic pressure in the presence of salt should be much less than when no salt is present. The experiments in Table III show that the latter results are obtained. 2 M NaSCN has very little effect on the viscosity of 10 per cent gelatin and the osmotic pressure decreases only slightly in the range from 37° to 3°C. The optical properties of the solution also bear out this as-

sumption, since a solution of gelatin at 37°C shows a very marked Tyndall cone, whereas the same solution in 2 M NaSCN shows only a faint cone

It has already been shown that the effect of temperature is largely on the insoluble component and it remains to be seen whether the effect of salt is also on this component or on the soluble fraction Table IV gives the effect of NaSCN on the osmotic pressure of a 5 per cent solution of the soluble and insoluble fractions at 37°C It is evident that there is a very large increase in the osmotic pressure of the insoluble fraction and only a small increase in that of the soluble one The salt therefore affects the same constituent as does raising the temperature

DISCUSSION

The preceding experiments appear to the writers to furnish a basis for a simple and satisfactory picture of the mechanism of the swelling of isoelectric gelatin in water and in neutral salt solutions The block of gelatin is a network consisting of threads of a substance insoluble in cold water and holding in its meshes a solution of a substance soluble in water (For the sake of simplicity only two substances are considered, although in reality there are probably a series of substances whose properties grade from very easily soluble to insoluble) The process of manufacture of gelatin would be expected to lead to the formation of just such a series of compounds, since it is formed by the hydrolysis of an insoluble substance, collagen Such a reaction would be expected to produce a series of split products ranging in complexity from the amino-acids up to unchanged collagen In the further process of purification and washing, all substances which can diffuse through the network of insoluble material are removed while those that cannot are held back When the block is immersed in water the internal solution exerts osmotic pressure and water is taken in until the osmotic pressure is equalized by the elastic force of the network A slow subsequent swelling will occur, however, owing to the fatigue of this elastic force A quantitative expression for the kinetics of swelling has been derived on this basis and will be discussed in a subsequent paper

Raising the temperature causes an increase in the concentration of

the soluble material and hence in the osmotic pressure of the solution and at the same time causes a decrease in the amount of insoluble material forming the fibers. The swelling, therefore, increases very rapidly with the temperature until above 35°C the network dissolves and the block disintegrates. The insoluble material, however, still exists in the solution in the form of particles capable of swelling, as shown by the pH viscosity effect, and containing a large amount of water, as shown by the form of the osmotic pressure-concentration curves and the viscosity curves. The addition of neutral salts to the solution acts in the same way as increasing the temperature, except that the material forming the network is broken up into smaller particles.⁹ In very high salt concentrations the reverse effect occurs and the gelatin eventually precipitates. The various peculiar hysteresis effects which have been noted in connection with the swelling of gelatin are referable to the effect of the conditions on the elasticity of the network, and are similar to those observed in any elastic body. Cooling the solution results in the reverse process. The particles or molecules of the insoluble material precipitate in the form of a network—if the concentration is high enough—enclosing the solution of the soluble material. If the concentration is low, this network separates in the form of small clots which still contain nearly all of the soluble fraction. It seems quite possible that the formation of this peculiar structure rather than of an ordinary precipitate is due to the presence of the soluble material which acts as a protective colloid, and that a pure preparation of the insoluble material would form a normal precipitate. A very similar condition occurs in the solution and repurification of casein, addition of small amounts of acid or alkali to isoelectric casein results in a normal saturated solution, as Cohn¹⁰ has shown. Precipitation of such a solution by back titration, however, gives a milky colloidal solution which may form a gel, so long as any of the casein remains in solution. When the isoelectric point is reached, where all the casein is insoluble, a normal precipitate is again formed.

⁹ This is perhaps analogous to the prevention of agglutination in strong salt solutions. In the case of bacteria it was found that a marked decrease in the cohesive force of the particles was noted in these high salt concentrations. Northrop J. H. and De Kruij P. H. *J Gen Physiol* 1921-22 iv 639

¹⁰ Cohn E. J. *J Gen Physiol* 1921-22 iv 697

This mechanism also accounts for the fact that gelatin and similar substances swell only in those solvents in which they are soluble, and it predicts that the greater the solubility the greater the swelling. The swelling which occurs during the early stages of the action of enzymes on gelatin also would be predicted since the hydrolysis increases the concentration of soluble material and hence the osmotic pressure. The swelling caused by enzymes is similar to the effect of salts or higher temperatures except that it is not reversible.

The alternative hypothesis that the effect of temperature or salts is to increase the hydration of the particles and hence the osmotic pressure seems less probable, since it requires that the hydration increase with increasing temperature. Since the hydration of gelatin liberates heat this would be contrary to Le Chatelier's principle. It also fails to predict the increased swelling obtained on adding the soluble fraction to the insoluble, since the osmotic pressure and viscosity curves show that the soluble fraction is less hydrated than the insoluble.

It is evident that the structure of gelatin outlined above is quite similar to that proposed by Hardy¹¹ and now widely accepted, except that at least two substances are postulated. The whole mechanism agrees in detail with the theory clearly presented by Duclaux¹² in connection with the swelling of rubber.

SUMMARY

1 A method is described for measuring the swelling pressure of solid gelatin.

2 It was found that this pressure increases rapidly between 15° and 37°C, and that the percentage change is nearly independent of the concentration of gelatin.

3 It is suggested that this pressure is due to the osmotic pressure of a soluble constituent of the gelatin held in the network of insoluble fibers, and that gelatin probably consists of a mixture of at least two substances or groups of substances, one of which is soluble in cold water, does not form a gel, and has a low viscosity and a high osmotic

¹¹ Hardy, W. B., *Z. physik. Chem.*, 1900, **xxxiii**, 327.

¹² Duclaux, J., *Bull. soc. chim.*, 1923, **xxxiii-xxxiv**, 36.

pressure The second is insoluble in cold water, forms a gel in very low concentration, and swells much less than ordinary gelatin

4 Two fractions, having approximately the above properties, were isolated from gelatin by alcohol precipitation at different temperatures

5 Increasing the temperature and adding neutral salts greatly increase the pressure of the insoluble fraction and have little effect on that of the soluble fraction

6 Adding increasing amounts of the soluble fraction to the insoluble one results in greater and greater swelling

7 These results are considered as evidence for the idea that the swelling of gelatin in water or salt solutions is an osmotic phenomenon, and that gelatin consists of a network of an insoluble substance enclosing a solution of a soluble constituent

THE EFFECT OF pH ON THE PERMEABILITY OF COLLODION MEMBRANES COATED WITH PROTEIN

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In a study of some of the factors affecting the formation of protein films on collodion membranes,¹ it was found that the amounts of protein adhering to the membranes varied with the pH in a way similar, in the case of gelatin, to the variation of the fluidity of the protein solutions with the pH. The connection between the viscosity of gelatin solutions and the swelling of gelatin particles had been clearly brought out by Loeb.² Dr Northrop suggested to the writer that it would be of interest to determine whether gelatin and other proteins, when deposited as films on collodion membranes, would exhibit a rise and fall of swelling in acid or alkali, as do granules of gelatin. This has been found to be the case.

EXPERIMENTAL.

The membranes were prepared on mercury by the method already described,¹ about 2 hours being allowed for the evaporation of the solvents through the cardboard case. These membranes were of the most permeable of the types studied in the previous experiments. They were cut into disks 3.81 cm. in diameter, and were coated with protein by being soaked overnight, at 30°C., in the protein solutions. In each case 3 disks were kept overnight at 30°C. in 25 cc. of the solution contained in a wide-mouthed 50 cc. Erlenmeyer flask, and in these experiments the flasks were not agitated. The disks from each flask were washed 3 times with about 300 cc. of water at 30°C. The

¹ Hitchcock D. I. *J. Gen. Physiol.* 1925-26 viii 61.

² Loeb J. *Proteins and the theory of colloidal behavior*. New York and London 2nd edition 1924.

weights of the disks, with and without protein, were determined after drying overnight at 100°C

The swelling of the protein on the membranes was studied by measuring the rate of flow, under known pressure, of water and other solutions through the protein-coated membranes in the apparatus previously used,¹ which is similar to that of Bartell and Carpenter.³ The membrane was bathed on both sides by the solution under investigation, which was forced through the membrane by a constant pressure of mercury. The area of membrane exposed to the liquid was circular, its diameter being 2.2 cm. The rate of flow was measured with the aid of a stop-watch and a mm scale, by observing the movement of a meniscus in a horizontal tube 0.0760 cm in diameter. The data were reduced to c.g.s. units and are given in terms of the permeability, Q , which may be defined as the number of cc of liquid flowing in 1 second through 1 sq. cm of membrane under a pressure of 1 dyne per sq. cm. The temperature varied from 19 to 24°C in different experiments.

It was found that solutions of HCl or NaOH up to 0.1 M flowed through membranes which had not been coated with protein at the same rate as water. Hence the differences observed with the protein-coated membranes are to be ascribed to an effect of the electrolyte on the protein rather than on the collodion.

Each experiment was carried out with a single disk of membrane, the determinations being made in the order of increasing concentrations of acid or alkali. Since the different membranes were not all alike, the data of the different experiments are not quantitatively comparable.

Table I shows the effect of different concentrations of HCl on the permeability of a membrane which had been soaked in a 4 per cent solution of isoelectric gelatin. At the end of the experiment 1.0 M NaCl was forced through the membrane, and this was finally displaced with water, which brought the permeability back nearly to the starting point. The experiment shows that while the acid solutions removed very little gelatin from the membrane, they changed the permeability in the direction to be expected if the effect of the acid were on the swelling of gelatin particles.

³ Bartell, F. E., and Carpenter, D. C., *J. Phys. Chem.*, 1923, xxvii, 252

Table II shows a similar experiment with NaOH. Determinations were not made in concentrations above 0.1 M because the alkali attacked the membranes so that they burst under pressure. The changes in permeability are again opposite in direction to the changes in swelling observed by Loeb.

TABLE I

Effect of HCl on Permeability of Gelatin-Coated Membrane

Untreated membrane, dry weight = 28.0 mg, $Q = 26.5 \times 10^{-10}$. At end of experiment, dry weight = 49.5 mg. Control without HCl treatment, 52.0 mg.

HCl, mols per liter	0	10^{-3}	3×10^{-3}	10^{-2}	3×10^{-2}	10^{-1}	3×10^{-1}	10^{-1}	3×10^{-1}	1		
$Q \times 10^{10}$	4.22	4.21	4.20	4.18	3.68	2.38	1.62	1.33	1.49	2.63	5.28	7.95

TABLE II

Effect of NaOH on Permeability of Gelatin-Treated Membrane

NaOH mols per liter	0	10^{-3}	3×10^{-3}	10^{-2}	3×10^{-2}	10^{-1}	3×10^{-1}	10^{-1}
$Q \times 10^{10}$	2.98	2.79	2.03	1.42	1.12	1.06	1.08	1.27

It was found by experiments with HCl and NaOH solutions containing also 0.1 M NaCl that the permeability of similar membranes could be altered by pH even in the presence of salt. Table III shows the results obtained with a gelatin-coated membrane whose permeability was measured in solutions of the glycine-phosphate-acetate buffer described by Northrop and De Kruijff.⁴ These solutions contained 0.125 M total electrolyte, the pH values were obtained with the hydrogen electrode after the solutions had been used for the permeability measurements. The experiment shows that even in the presence of salt the pH exerts a considerable influence on the permeability, the latter being greatest in the vicinity of the isoelectric point of the protein.

⁴ Northrop, J. H., and De Kruijff, P. H., *J. Gen. Physiol.* 1921-22, iv, 639.

TABLE III

Effect of Glycine-Phosphate-Acetate Buffer (0.125 M) on Permeability of Gelatin-Coated Membrane

Untreated membrane, dry weight = 27.0 mg, $Q = 25.7 \times 10^{-10}$ At end of experiment, dry weight = 49.6 mg Control without buffer treatment, 48.0 mg

pH	3.45	3.85	4.16	4.95	5.18	5.55	5.97	6.42	6.94	7.41	8.04
$Q \times 10^{10}$	1.63	1.71	2.27	2.47	2.27	2.14	1.86	1.86	1.78	1.74	1.69

Measurements of the permeability of a gelatin-coated membrane in several solutions of HCl and H₂SO₄ showed that the permeability was decreased less by H₂SO₄ than by HCl when the comparison was made at equivalent concentrations. The changes were of the same general nature as those in Table I, but the permeability in H₂SO₄ was always somewhat higher than in HCl. This result is qualitatively in accord with the experiments of Loeb² on the swelling of gelatin in acids, although the difference between the effects of the two acids appears to be less in the case of permeability than in the case of swelling. Another membrane was tested in a similar way with NaOH and Ba(OH)₂. Again the result was qualitatively similar to the swelling experiments, the permeability being always higher in the presence of a divalent ion of opposite charge to that of the protein.

In order to determine whether the effects observed were confined to gelatin-coated membranes, a few determinations were made of the permeability in HCl of membranes which had been soaked in solutions of egg albumin, edestin, euglobulin from ox serum, and serum albumin from horse serum. A single disk coated with each protein was used for permeability measurements in HCl of the concentrations 0.001, 0.01, and 0.1 M. The amounts of adherent protein on the membranes used were 28 mg of egg albumin, 4 mg of edestin, 15 mg of globulin, and 11 mg of serum albumin. In every case the permeability was lowest in 0.01 M HCl. The egg albumin and edestin showed only small differences, but in the case of the serum proteins the effect was larger. With serum globulin the permeability in 0.01 M HCl was about two-thirds of that in the 0.001 or 0.1 M

HCl, while with serum albumin and 0.01 M HCl the permeability was less than half of the value obtained with the other two solutions.

SUMMARY

The permeability of gelatin-coated collodion membranes, as measured by the flow of water or of dilute solutions through the membranes, has been found to vary with the pH of the solutions. The permeability is greatest near the isoelectric point of the protein, with increasing concentration of either acid or alkali it decreases, passes through a minimum, and then increases. These variations with pH are qualitatively in accord with the assumption that they are due to swelling of the gelatin in the pores of the membrane, the effects of pH being similar to those observed by Loeb on the swelling of gelatin granules. Indications have been found of a similar variable permeability in the case of membranes coated with egg albumin, edestin, serum euglobulin, and serum albumin.

TEMPERATURE CHARACTERISTICS FOR SPEED OF MOVEMENT OF THIOBACTERIA *

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I.

The gliding movements of certain *Cyanophyceæ*, *Thiobacteriales*, and gregarines provide relatively uncomplicated types of comparable activities suitable for quantitative observation. In spite of much discussion little is known as to the mechanism of this sort of progression. Interpretations have been advanced by a number of writers¹ but data required for formulation of a theory of this sort of movement have been lacking. For the present, we are not concerned so much with the mechanics of the movements of *Oscillatoria*, *Beggiatoa*, and other forms creeping in ways apparently similar, but in employing the rate of this type of movement as an index of metabolic changes.

For one kind of *Oscillatoria* it has been shown (Crozier and Federighi, 1924-25) that the rate of translatory movement, in this instance uncomplicated by rotation, obeys the Arrhenius equation for change with temperature. The rates of movement at different temperatures permit evaluation of the constant E , or μ , in the equation

$$\text{Velocity} \propto e^{-\frac{\mu}{RT}}$$

where e is the Napierian base, R the gas constant, and T the absolute temperature. The value of μ obtained in the experiments cited was 9240.

In these observations the light intensity was practically constant.

* Support from the Milton Research Fund of Harvard University is gratefully acknowledged.

¹ For recent views consult Fechner (1915) Schmid (1918 1923) Prell (1923 a and b) Krenner (1925)

To discover the way in which the magnitude of E might be dependent upon light intensity, it was desired to know, among other things, the relations between speed of movement and temperature in a form free from effects due to photosynthetic activity. For this reason, in part, we turned to the leuco-thiobacteria. It is found that the relation of motility to temperature in *Beggiatoa* and *Thiothrix*, as in *Oscillatoria*, points clearly to the controlling influence of chemical processes. The values of the critical increments (E , or μ) in fact agree sufficiently with those repeatedly obtained for other activities of various organisms, and specifically for catalyzed respiratory oxidations (*cf* Crozier, 1924-25). The value of E derived for speed of movement in *Beggiatoa* thus emphasizes the fact that the magnitude obtained with *Oscillatoria* (Crozier and Federighi, 1924-25) does not accord with any commonly encountered (Crozier, 1925-26, *b*) in connection with biological processes adequately studied. The investigation of the relation between E and light intensity is therefore expected to yield interesting suggestions as to the significance of the critical increment for movement in the case of *Oscillatoria*. This will be discussed in a subsequent paper. In the meantime, it may be pointed out that the movement of *Beggiatoa* appears to be governed by chemical processes similar to those revealed in a number of other vital activities, the details of the relationship between speed of translatory movement and temperature show certain features of general interest for this method of analysis.

II

The organisms employed for the measurements were kept in shallow culture dishes containing the usual variety of forms occurring in brackish water putrefactive sulfureta (*cf* Bavendamm, 1924, Baas-Becking, 1925). Two species, identified as corresponding to *Beggiatoa alba* and to a species of *Thiothrix* (*T. tenuis* ?), were taken for study from particular spots in one culture. Thin smears were mounted between two cover glasses, the lower one small enough to be placed within the glass ring of a van Tieghem cell. The cell was sealed with paraffin or chicle, and had in it a small volume of the culture liquid. The sealing was necessary to prevent dilution when the preparation was submerged in a thermostat, access of tap water

caused cessation of progression movements. The cell was mounted in a mechanical stage on the platform of a microscope so adjusted as to have the preparation submerged to a depth of 10 cm. in a water thermostat. The mechanical stage and the fine adjustment of the microscope were controlled by suitable attachments projecting above the water level. With good stirring no difficulty was experienced in maintaining desired temperatures. Light from a housed tungsten bulb was reflected from a mirror beneath the microscope. Variations in light intensity were apparently without effect on the movements of the sulfur bacteria, but for practically all of the measurements the light was of approximately 30 m c intensity. Within periods of 6 hours or longer, even up to 24 hours, no progressive changes in speed of movement were detected. Hence the sealed atmosphere in the observation cell produced no special effect.

The measurements were made of the longitudinal progression of straight filaments and so far as could be determined in the absence of mechanical impedance. With a 5 mm objective and 7.5 \times ocular, ten divisions of the ocular micrometer used corresponded to 0.05 mm. The time required for each filament to traverse this distance was taken with a stop-watch a number of readings being secured at each temperature. With each preparation used, precautions were taken, through time records and by reversing the sequence of temperature changes, to insure the absence of irreversible thermal effects.

III.

According to the current understanding of the mechanism of movement in *Oscillatoria* the longitudinal membrane (Hinze 1902) of a filament is pierced by pores, through which a carbohydrate mucus is extruded (Fechner, 1915, Schmid 1918, 1923, Prell 1921 a Krenner, 1925 Ruhland and Hoffmann, 1925). This would account for the phenomena which gave rise to the older conception of "extracellular protoplasmic streaming". Another view regards the movement as due to "modifications of surface tension," perhaps caused by osmotic processes (Coupin 1923) though suggestive, no particularly relevant evidence supports this idea. Krenner (1925) found the speed of transitory movement of *Oscillatoria* to vary inversely with the diameter of the species and that the osmotic pressure of the proto-

plasts is higher in the narrow forms (measured by plasmolytic shrinkage method) Krenner therefore supposes that the specific speed of motion is determined by the turgor For *Oscillatoria* and its relatives it is known that in general the stouter forms are the more slowly moving On this basis, one might rather expect the specific speed to be determined by some relationship of surface to bulk But among the sulfur bacteria we find that with forms occurring side by side in the same culture, the larger species move more quickly,—for two forms, in about the ratio of 1 to 1.5, at the same temperature, when the filament diameters are in the ratio 3.22:1

We are by no means clear as to the meaning of the optical evidence for “extracellular protoplasmic streaming” (*cf* also Crozier and Federighi, 1924–25, and Krenner, 1925), nor as to the homology of the superficial slime-covering in *Beggiatoa mirabilis* (Hinze, 1902, Ruhland and Hoffmann, 1925), which we have also observed, with surface structures in the forms we have employed for measurements of speed of movement

According to Schmid (1923), who studied fragmented filaments, all parts of a filament of *Oscillatoria* are motile Prell (1921*a, b*,) found that the cells of a filament “cooperate,” although there seems to be no conduction of stimuli from one part of a filament to another This agrees with the observation (Crozier and Federighi, 1924–25) that the speed of movement does not vary with the length of the filament Mr E. S. Castle has made similar observations on *Anabaena* It has been noticed, however, that very small groups of cells do not move (Krenner, 1925) In *Beggiatoa* very short fragments, even comprising but three to five cells, do move, but only for very short distances, the frequency of reversal in direction is very high It is to be noted, as bearing upon unity of action in long filaments, that there is frequently apparent a failure of the parts of a filament to cooperate With very long filaments (2 mm), the two terminal regions may be moving in opposite directions, or a hook bend at one end may be moved forward bodily, in such fashion as to indicate that the bent tip region is not at all contributing to the movement Similar cases occur in which reversal of direction of movement is not synchronous over the whole filament (*cf* also Keil, 1912) Aside from their bearing upon the mechanism of movement, these points are of practical moment for

the measurement of speeds of progression under comparable conditions

The speed of movement declines as the culture containing the thio-bacteria ages and the cells of the organisms become vacuolated. During the most active period of growth the speed of translatory movement is quite sufficiently uniform to permit significant measurements. The speed is independent of the length of the filament. Successive estimations with a single filament show satisfactory constancy, as may be illustrated by several sets of readings

Filament.	Temperature, °C	Time to travel 10 micrometer divisions, sec.
A	10.2	34.6
		38.6
		39.8
		39.0
		36.8
B	16.8	23.8
		22.2
		21.8
		22.1
C	19.3	14.4
		17.6
		17.2
		18.0

It could not be shown that these slight variations are related to the incidence of reversals of direction.

The latitude of variation in such series does not significantly differ from that in series obtained from a number of different filaments. This was tested sufficiently to ensure the possibility of employing averages based upon measurements with a number of filaments. It is practically impossible, however desirable to obtain readings over a range of temperatures from single filaments. There is indication of fluctuating variation in speed of movement not correlated with time of day, in which the period is rather long. This is in part responsible for the scatter of the plotted means (Fig. 1). The probable error of the plotted means is less than 5 per cent of the corresponding means.

(usually less than 4 per cent) For purposes of the present account we have employed data from filaments in one culture, between January 14 and January 28, 1926 Throughout this period no systematic changes in speed of movement were detected The number of observations was 431

IV

The results are plotted in Fig 1 Contrary to the case of *Oscillatoria* (Crozier and Federighi, 1924-25), the motion of *Beggiatoa* ex-

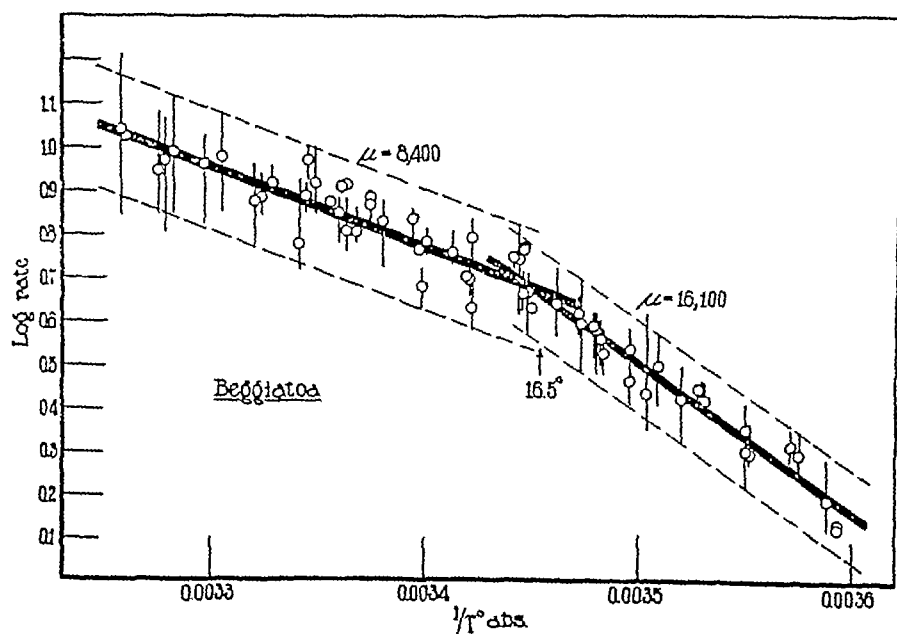


FIG 1 Speed of gliding motion of *Beggiatoa* as function of temperature The circles give positions of average speeds in series of readings, the vertical lines extending from them cover the latitude of variation in each series The rate is taken as 100/ time to travel 10 micrometer divisions (0.05 mm)

hibits a sharp change in the relation to temperature at about 16.5° The lines providing a satisfactory fit to the two portions of the log speed- $1/T^{\circ} \text{ abs}$ graph have slopes respectively, yielding $\mu = 8,400$ and $\mu = 16,100$ as temperature characteristics

It may be suggested that the data are equally well fitted by a single unbroken curve To this there is definitely opposed the fact that in such a case the slopes of the fitted straight lines could not very well be

expected to agree with those found in numerous cases where a single rectilinear relationship holds over the whole of the temperature scale. The impossibility of describing such series of observations by means of a single smooth curve is adequately shown by plotting rates against centigrade temperatures, the points fall upon two sharply intersecting curves. And there is also to be emphasized the fact that the temperature at which intersection of the proposed straight lines is located, as determined solely by the distributions of the relevant points, agrees so closely with one at which such irregularity is commonly or very fre-

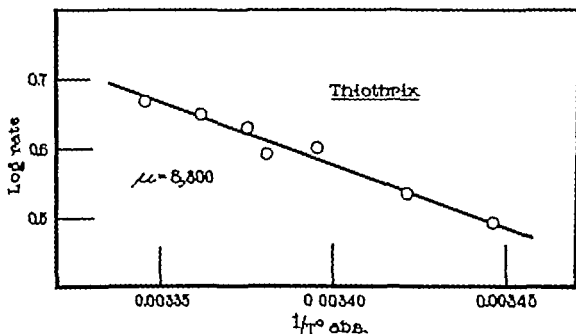


FIG. 2. Speed of movement in *Thiothrix* as related to temperature. The points are averages of 6 to 8 measurements each.

quently manifest in other vital processes (Crozier, 1925-26, b). Additional considerations justifying this procedure are discussed in another place (Crozier and Stier, 1926-27). Less extensive observations on the movement of *Thiothrix* provide data for the graph in Fig. 2. The temperature characteristic, $\mu = 8,300$, agrees well with that for the corresponding temperature range with *Beggiatoa*.*

* As with *Oscillatoria*, question also arises here as to the character and mechanism of reversal in direction of movement. According to Coupin (1923) *Oscillatoria* filaments, on Knop medium to which gelose had been added, show no regular periodicity in the reversal of movement but it is necessary to maintain constant conditions of light and temperature before the matter can be tested. It is clear

In addition to the occurrence of a critical "break" at 16°, the temperatures 5.3° and 33° were established at points at which progressive slowing of movement with time becomes evident, at 33° or above "jerky" side to side movement is evident, with little forward motion.

The "break" at 16° is made obvious in another way. The latitude of variation at temperatures below 16° is definitely less than at higher temperatures. For some time it has been desired to find instances in which it might be possible to discover if the latitude of variation is a property of the organism or tissue as a whole, or of the process whose critical increment is being measured. It is clear, we believe, that in general, and depending on the nature of the activity considered, both these types of variation must be recognized as possible. In many instances it has appeared that the latitude of variation may change without affecting the temperature characteristic (e.g., Crozier and Stier, 1925-26, 1926-27), on the other hand, the latitude may be sensibly constant when the increment changes. The present case is one in which there is apparent alteration of the latitude accompanying a change of increment.³

V

SUMMARY

The speed of translatory movement of *Beggiatoa alba* is governed by temperature in such a way that between 5° and 33° the temperature characteristics $\mu = 16,100$ and $\mu = 8,400$ respectively obtain for the temperature ranges 5° to 16.5° and 16.5° to 33°. The "break" at 16°-17° is emphasized by the occurrence of a wider latitude of variation in speed above this temperature. Above 16° the progression of *Thiothrix* yields $\mu = 8,300$. The possible relation of these values to that previously obtained for similar movement in (photo-synthetic) *Oscillatoria* is commented upon.

that the frequency of reversal is related to the length of the filament, being greater with short filaments, and certainly it increases with elevation of temperature. Reversal is more frequent in *B. alba* than in *Thiothrix*, under the same conditions. In forms we have observed the frequency of reversal has a higher temperature coefficient than the speed of translation.

³ This may also be the case with the locomotion of *Paramecium* (Glaser, 1925-26).

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STEREOTROPISM IN RATS AND MICE

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I.

With diplopods (Crozier and Moore, 1922-23) and with larvæ of *Tenebrio* (Crozier, 1923-24, a, b) it has been shown that posterior unilateral contact of the creeping animal with the edge of a thick glass plate forces the head to turn in the direction of contact. Contact with two lateral surfaces of equal extent prevents stereotropic bending, and the animal proceeds in a straight path. It was also found, especially with *Tenebrio* larvæ, that stereotropic orientation due to unilateral contact, particularly at the anterior end, persists briefly after cessation of the contact, and that unequal bilateral contacts lead to orientation through an angle roughly proportional to the difference in areas of contact.

These observations can be repeated with a variety of forms, land isopods among others, and show the purely tropistic character of such orientations during creeping. The present experiments are concerned with rats and mice. In all essential details the results duplicate those obtained with arthropods. Their special interest lies in the fact that they demonstrate the occurrence of simple tropistic behavior in mammals. The effects of memory images (Loeb 1918), and of the great enrichment of sensory fields and their central projections (Parker, 1922), enormously increase the number of possible responses which higher vertebrates may exhibit, and prediction of the course of movements is correspondingly restricted. In order to observe tropistic conduct in a mammal it is necessary to deal with a type of response which dominates the animal's conduct so strongly as to exclude the influence of stimulations not directly connected with this particular aspect of behavior. The stereotropism of young rats and mice fulfill* this condition.

The behavior of the young opossum at birth appears to give an instance of geotropism. After being licked free of blood and embryonic membranes, they climb "hand over hand" from the genital opening to the pouch (a distance of about three inches), and attach themselves to the teats (Hartman, 1920). "If the skin be tilted, the embryos can be made to travel upward and even *away* from the pouch for they are negatively geotropic." The postural reflexes of the decerebrated rabbit (Magnus, 1915-16) include responses which may be taken to have a basis in stereotropism. If placed on the ground with the body in an asymmetric position, the head moves to the normal symmetrical orientation even in the absence of otic labyrinths. This is prevented if a board is placed upon the animal lying in an asymmetrical position, when asymmetric contact-stimulation is equalized, the animal retains an abnormal position.

Accounts of the behavior of rats and mice (e.g. Vincent, 1911-12) contain a number of observations suggesting stereotropic guidance.¹ We desired to see if tropistic conduct could not be demonstrated more clearly. This is best accomplished by determining if, as in the case of invertebrates, stereotropic orientation during creeping might not obey the law of the composition of forces. In this event equal bilateral contacts should obliterate turning toward a source of contact. To rule out effects of vision and of the tactile rôle of vibrissæ, we have used animals lacking eyes or vibrissæ. It is neater, in so doing, to avoid experimental mutilation by employing individuals "operated upon" through the agency of a genetic factor, such as results in blindness. This we have been able to do.

II

The animals used were young rats (*Rattus norvegicus*) and mice (*Mus musculus*) aged 9 to 20 days. The rats were albinos and dark-eyed young from a backcross of the King inbred albinos to hooded rats sired by King inbred males. The mice were all dark-eyed.

¹ Watson (1914, p. 424) seems to express a view generally held, that "the so-called 'stereotropism' which such animals exhibit is probably no more a case of stereotropism than is the action of a blind man in keeping near a wall or the edge of the side walk." On the preceding page, moreover, he considers it "strange" that when vibrissæ are removed from the *right* side, the rat keeps close to the *left* side of the path in a maze. Cf. also Przibram (1913, p. 99).

The eyes of young mice and rats are opened 10 to 14 days after birth. At first, only animals with unopened eyes were used, but it was soon found that young animals with opened eyes gave substantially the same results. Tests with rats were for the most part made in a dark room, under red light of low intensity, at 23–25°C. Experiments with mice were made at about the same temperature, but not in a dark room. Temperatures as low as 15° greatly reduce activity.

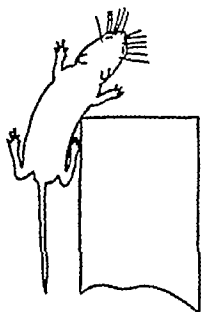


FIG 1

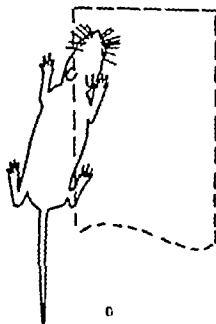


FIG 2.

FIG 1 Stereotropic orientation of young rat or mouse at the corner of a box along one side of which it has been creeping

FIG 2 A young rat or mouse has been creeping in contact with the side of a box (dashed outline) the removal of the box results in partial orientation toward that side

Typical stereotropic behavior is observed in animals creeping or walking at a fairly rapid rate. During slow progression there is more opportunity for the lurching gait to induce movements which, while in the main of stereotropic origin, nevertheless interfere with diagrammatic orientation.

Contact with a vertical surface during creeping results in its being followed closely, and at the end of the surface bending is invariably seen toward the contact side. Depending upon the rate of creeping, the animal either proceeds at an angle with the path while in contact,

or if the progression has been slow, it may turn and continue to maintain contact Fig 1 shows the path taken after contact with the side of a box, and at its corner If the box is suddenly removed while the animal is creeping, there is always a swerving toward the side where the box was located (Fig 2) Stroking one side causes turning in that direction These results are exactly similar to those gotten with arthropods

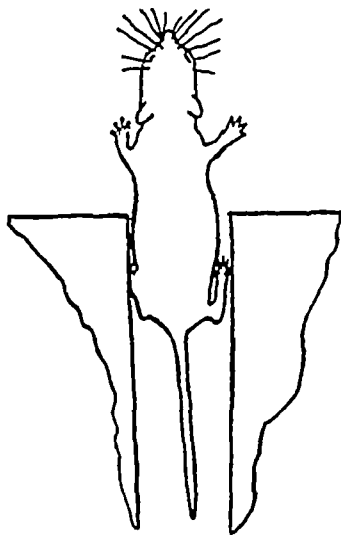


FIG 3 A young rat or mouse creeping in a passage-way between two boxes, just wide enough to permit gentle contact on either side during the animal's swaying progression, is found to emerge from the passage-way without orientation Equivalent bilateral stimulations prevent stereotropic turning

When the vibrissæ have been recently cut away the creeping movements are slower and more uncertain, yet the stereotropic responses continue, several days later the uncertainty of the creeping is lost, but the animal continues to move with head held close to the floor Removal of the tail has even less effect Removal of both tail and vibrissæ does not materially interfere The surface of the body and legs is thus sufficient to control stereotropism

III

A young rat or mouse creeping between two boxes so placed as to give equal contact on either side typically emerges from the alley-way

in a perfectly straight course (Fig 3) The bulging of the body at the level of the hind legs, coupled with the lurching gait, sometimes causes quite unequal contacts on the two sides, and this results in modification of the path on emergence If one box is advanced beyond the other, the animal frequently emerges at an angle toward the extended

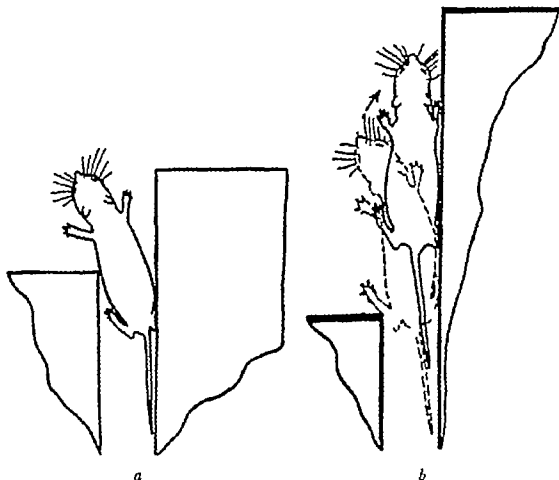


FIG 4 *a* Contact at one side with the corner of a box may lead to orientation toward that side, apparently due, in part at least to more intense tactile excitation than is provided by a continuous flat surface (or by smoothly rounded corners, see Fig 5)

b When such a corner is passed, orientation persists toward a continuing contact on the opposite side.

side This angle decreases with increase of the excess contact zone on that side If one surface extends more than the length of the body beyond the corner of the opposed box, the mouse or rat emerges at a very acute angle and then orients so as to round the corner against the surface of the more extended box (Fig 4) This is not exactly the

result obtained with arthropods and other forms, which emerge at an angle toward the side of more extensive contact. But further tests show that this at-first-sight anomalous result is due to the relatively excessive tactile stimulation provided by the sharp corners of the wooden boxes used in such experiments. The same outcome is generally observed if thick blocks of paraffin are used instead, provided they have

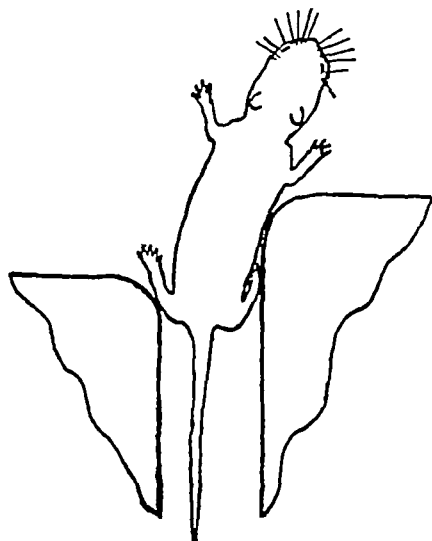


FIG 5

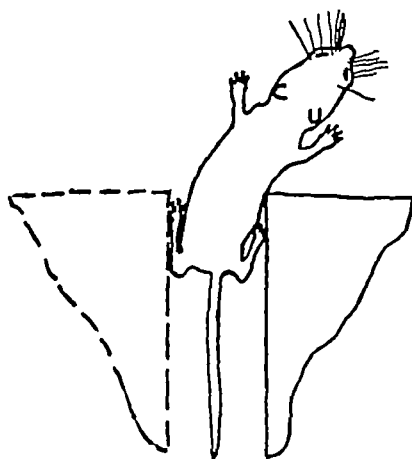


FIG 6

FIG 5 When blocks providing lateral contacts are of unequal extent, the young rat or mouse orients toward the side of more extensive contacts, but does not completely turn the corner unless the difference in extent of the two blocks is more than half the length of the animal. This, the expected result from a tropistic standpoint, is obtained when the corners of the contact blocks are smoothly rounded.

FIG 6 An individual emerging from equal bilateral contacts with two boxes (*cf* Fig 3) proceeds in a straight path, without orientation, but if one of the boxes be removed (dashed outline), it promptly orients toward the remaining one.

sharp corners. But if the corners be smoothly rounded, the result of such tests is entirely consonant with the interpretation that stereotropic orientation during creeping varies in amplitude according to the difference in the excitations on the two sides (Fig 5). If one of two opposed boxes providing bilateral contact at emergence be suddenly removed, the animal orients toward the remaining surface (Fig 6).

These experiments were repeated many times, with particular effort to obviate any persistent tendency of single individuals to right or left hand turning

During creeping in contact with a single vertical surface, the young rat or mouse, especially if moving very slowly, occasionally reverses direction. Observation shows that this occurs when the opposite side makes contact with the floor, as the animal falls into the "corner" between the floor and box, this is similar to the rotation of the body on its long axis observed with invertebrates creeping in the angle between a vertical and a horizontal surface. As a rule, the area of contact is then greater on the side toward the box, and orientation in this direction results in reversal of the path

IV

These responses have also been obtained with adult mice and rats, but visual and other sources of stimulation frequently make them much less precise

Mice blind through hereditary defect characterized by absence of visual cells in the retina were very kindly loaned to us by Dr Clyde Keeler of the Bussey Institution (*cf* Keeler, 1926). The locomotion of these adult mice is much more direct than in the case of the very young individuals, and the typical stereotropic responses were obtainable with great certainty and clearness

V

The reactions we have described as typical are of course not exhibited with diagrammatic clearness at every trial. The more significant sorts of deviation, however, are themselves stereotropic in origin

The stereotropism of rats and mice as observed in these experiments was always positive. Movement away from a contact surface is occasionally seen with the younger animals, but it is easily shown that this is an accidental consequence of the method of creeping. The leg muscles are not yet well developed and the legs are disproportionately long. The body is kept fairly close to the ground and the legs are advanced in a way which cause the rather unsteady creeping act to be a

succession of pronounced lurches. An occasional lunge removes the animal from a vertical contact surface, and if all contact has been lost it may creep away from it, usually, however, the residual effect of the contact surface is sufficient to cause reorientation toward it. In case complete separation from the vertical surface has not been followed by reorientation and return, test by contact with a new surface always shows that the animal is still positively stereotropic.

The swaying mode of progression may cause a young rat emerging from bilateral contacts to move toward one side. Thus in one series of trials, with five rats aged 20 days, each animal passed eight times between two vertical contact surfaces of equal extent, in another series eleven rats aged 12 to 14 days each passed five times through equal vertical contact zones, in thirty-one of the first forty tests, and in forty-four of the second lot of fifty-five tests, emergence was in a straight line, in the cases of deviation toward one side, it was seen that the divergence was due to a lunging in that direction rather than to an act of orientation.

When the animal moves at a fairly rapid rate the unilateral effects of lurching movements are more or less equalized. With older individuals the stronger legs make for a straighter course and the influence of lurching motion almost completely disappears. Thus animals about 25 days old show extremely regular reactions, as do the adult blind mice.

The chief sources of apparent irregularity in the stereotropic responses is found to lie in chance contacts of tail and especially of vibrissæ with the boxes employed to give contact surfaces. These variations are reduced by removal of vibrissæ and tail, but the slowness and uncertainty of progression subsequent to these operations introduce other complications and prevent precise measurement of the relation of unequal contact on the two sides to the angle of orientation.

VI

SUMMARY

Typical stereotropic orientation toward a lateral surface of contact is obtained in young rats and mice, and with adult mice congenitally blind. Removal of vibrissæ or tail or both does not essentially affect this response.

Equal contact on both sides of the body prevents orientation toward either source of contact. Unequal contact areas on the two sides leads to orientation toward the more extensive contact.

This behavior very exactly parallels the stereotropic conduct of arthropods, and thus provides a fairly complete instance of a tropism in mammals.

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HUMAN GROWTH CURVE

By CHARLES B. DAVENPORT

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(Accepted for publication, July 26 1926)

1 Statement of Problem

The fascinating changes in the velocity of development, as well as in proportion of parts, that the growing child shows have long been watched with interest by parents but have only lately been made the subject of scientific analysis. Quetelet (1870) was perhaps the first to measure children at each year of age, but his subjects were few in number (ten to each year), strictly, but not always wisely, selected. Then came the measurements of great numbers of school children by Bowditch (1875) in Boston. This work was followed by a host of similar investigations whose results are summarized by Baldwin (1921) and in my 1926 paper.

The first attempt to interpret the course of human development on a chemical basis was made in the same year by W. Ostwald and by T. Brailsford Robertson, the latter of whom has published a remarkable series of papers since 1908. Robertson early concluded that there are three maxima in the curve of growth of man, one intrauterine, a second that reaches its greatest velocity at about the 6th year, and a third which, in the male, occurs at about the 16th year. This view of a triple set of growth cycles is still adhered to by Robertson who discusses them fully in his book *Chemical basis of growth and senescence* (1923) and later papers. Robertson's conclusions have been largely based on the data published by Quetelet, and this selection has not been altogether fortunate. Brody has extended Robertson's methods of analyzing the growth curve, but recently (1926) he has found the human growth curve to be of a different order from the growth curve of other mammals and he has been led to abandon, for the present, attempts at its interpretation. In view of the un-

satisfactory condition of the analysis of the human growth curve it has seemed desirable to reattempt it, using the best available data. This is the excuse for the present paper.

2 *Methods and Material*

The curve of development of weight from conception to maturity (Fig 1) is based on data drawn from various sources. For the antenatal portion the data of Streeter (1920) have been utilized. For postnatal weights, up to 6 years, the data of Woodbury (1921) have

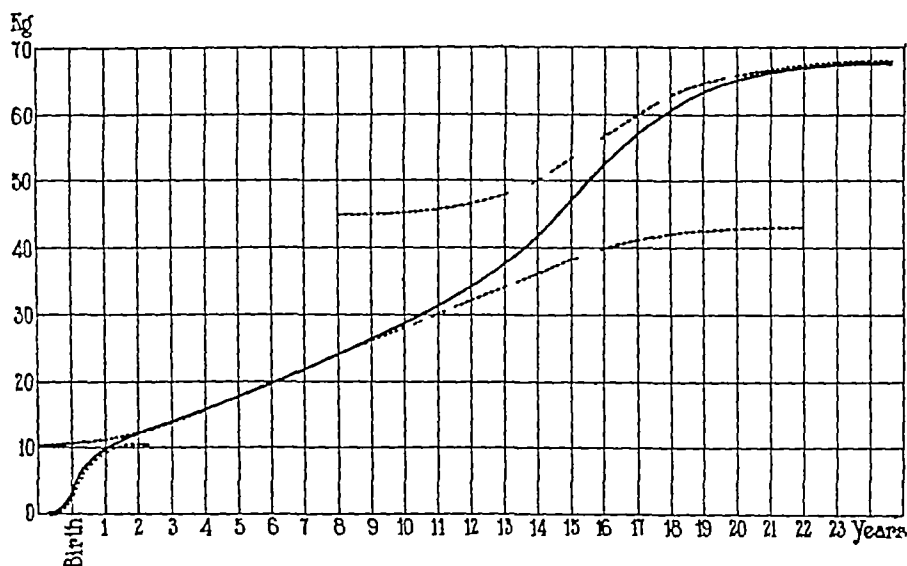


FIG 1 Analysis of the developmental curve of body weight (full line) into two auto-catalytic curves (dotted line at top and bottom) and a residual curve (dotted line in the middle). Human Nordic stock, males. The autocatalytic and residual curves drawn in free-hand. Abscissæ, time in years, ordinates, body weight in kilos.

been used. For later years various sources, chiefly Nordic males as given in Table B of my *Human metamorphosis* (1926), were used.

For annual increments in weight (Fig 2) the same sources have been used, together with my Table D (1926) for Nordic males.

The dotted curves of Fig 1 were put in free-hand to indicate the location of possible autocatalytic curves. The dotted curves of Fig 2

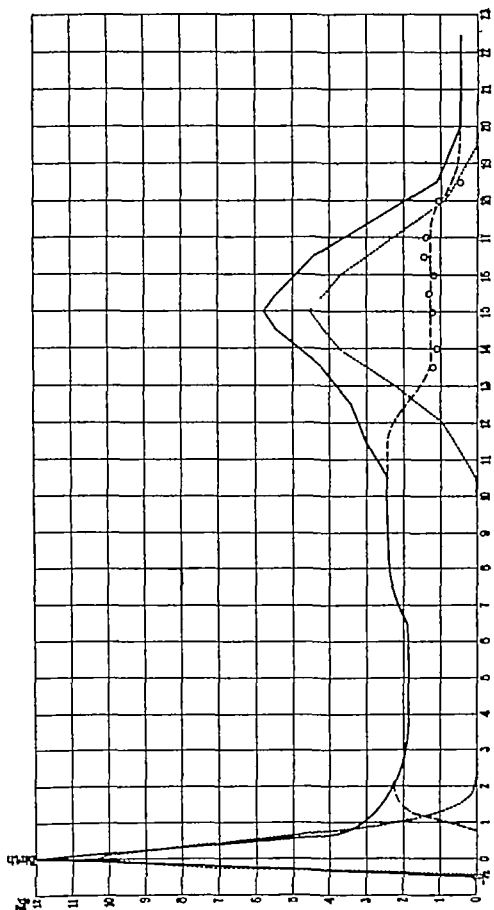


FIG. 2 Full line, the curve of annual increment rate of total body weight, male Nordic stock (see text) Dotted line to left, theoretical skew curve corresponding to increments of circumnata growth cycle. Dotted line to right, theoretical "normal" curve of increments corresponding to adolescent growth cycle. Dash line 0.75 to 2.25 years, full line 2.25 to 10.5 years dash line 10.5 to 20 years, and full line beyond 20 years indicates the residual growth increments. Abscissa time in years, ordinate, annual rate of increments in weight in kilos. Small circles between 13 and 19 years indicate the precise points upon which is based the position of the dash line, which is smoothed between these years.

were drawn after careful computations, as described below All statistical work was checked

The curves are plotted on arithmetical paper, instead of logarithmic paper as is frequently done The justification for the latter practise is found in the theoretical conception, clearly expressed by Minot (1891) "The increase of weight depends upon the amount of body substance or, in other words, of growing material present at a given time" As I pointed out many years ago (1897)¹ not all the body substance is "growing substance" During early development much water is imbibed which adds to the weight of the body, and although it may accelerate growth is not itself "growing material" During later development "body substance" is being laid down as formed substance that has primarily a mechanical or sustentative function and is not growing material Indeed, a consideration of the complex processes of growth leads to the conclusion that to plot growth on logarithmic paper leads to just as great a distortion of the facts as to plot it on arithmetical paper Since the latter method of plotting has the merit of simplicity, I am adopting it in this paper and suggest its uniform adoption until the advantages of some other method of plotting growth curves shall have been demonstrated

3 RESULTS

The arithmetical curve of growth, as plotted in Fig 1, begins near 0 kilos at the time of fertilization of the egg It increases slowly at first, then with ever accelerating velocity, until at birth it is shooting upward at its steepest angle After birth the angle of slope gradually diminishes to the age of 2 or 3 years It runs upward at a tolerably uniform rate until 7 or 8 years of age, then begins to rise more rapidly again, reaches a second maximum of slope at 14 or 15 years and then gradually approaches the horizontal

If one contemplates this curve of growth one is struck by the resemblance of its two ends to the autocatalytic curve, to whose importance for growth Robertson has so forcefully called the attention of biologists I have drawn in, free-hand by dotted lines, such approximate autocatalytic curves But the growth curve, as a whole, is very

¹ Davenport (1897), pp 82 and 83

far from being merely one, two, or three autocatalytic curves. The analysis of the curve may be made more readily if we transform it into a curve of growth velocities, and this is done in Fig 2, which also is drawn on the arithmetical scale. The curve thus drawn is an instructive one. Starting at the zero base line the velocity of increments in weight rises, at first slowly, then more and more quickly, to a striking peak which corresponds with the moment of birth. At this time the child is increasing at the rate of 12 kilos per annum. The absolute velocity of growth at this period is greater than at any other time during life.

After birth the velocity of growth proceeds to diminish just as rapidly as it had increased before birth. This leaves out of account the cessation of growth that is well known to occur during the 2 or 3 days after birth, since to consider it would unnecessarily complicate the main result. The curve of velocity of growth in weight runs nearly on a level from $3\frac{1}{2}$ to $6\frac{1}{2}$ years. It then rises very gradually for a year or two, remains constant from $8\frac{1}{2}$ to about 10 years, and then proceeds to climb up to a second peak which it reaches at 15 years (in the male), at which time there is an annual growth increment of about 5.75 kilos per annum. After this peak is reached the velocity of growth diminishes to 20 years and then continues at an annual rate of slightly less than 0.5 kilos to middle life. The curve does not reach zero, on the average, because the population of adult males in the United States gains about 1 pound a year from 22 to 26 years and $\frac{1}{2}$ pound thereafter until about 45 years and about $\frac{1}{4}$ pound from then until 55 years of age.

Our velocity curve brings out clearly the fact that growth is not one autocatalytic process. It suggests the hypothesis that there are two autocatalytic cycles, one that we may call the circumnatal and the other the adolescent. The circumnatal cycle begins at fertilization of the egg, reaches a maximum at birth and probably ends at between 2 and 3 years. An attempt to fit a theoretic curve to this cycle has been made. The best fit is given by a skew curve of Pearson's (1895) Type I. Its formula is

$$y = 854.9 \left(1 + \frac{x}{5.221}\right)^{0.983} \left(1 - \frac{x}{38.876}\right)^{7.134}$$

This curve is plotted by a dotted line in Fig 2 Its standard deviation is only 5.17 months The y_0 value is 854.9 gm per month or 10.259 kilos per year This falls short of the empirical value of 12 kilos per annum partly because smoothed values were used in computing the theoretical curve The modal velocity of 12 kilos at birth is based on Zangemeister's data (1911), which are at least conservative in respect to velocity of growth at birth

The circumnatal curve of velocity is, as stated, a skew curve of Pearson's Type I The index of skewness is 0.31 In the formula given above the denominator of the fraction in the second factor gives the range in months of that part of the curve that lies between birth and conception It is 5.22 months The denominator of the fraction in the third factor gives the range in months of that part of the curve that lies between birth and the end of the circumnatal growth spurt, amounting to 38.88 months The theoretical range to the left of the mode is thus only 5.2 months, while, actually, development begins at 9 months before birth However, at the end of the 6th month before birth increments in weight are only just becoming considerable (10 gm monthly) so that the calculated curve agrees here fairly well with the observed curve The other end of the curve is at about 39 months, at which age the observed curve of increments has nearly reached the bottom of its first peak Thus the theoretical and observed curves are in close agreement

The adolescent spurt is probably measured by a normal frequency polygon, whose mode at 15 years in the male corresponds with that of the total increment curve It seems to start at about the 10th birthday and ceases at the 20th The adolescent episode of growth thus extends over 10 years or from 10 to 13 per cent of the full span of life

The theoretical normal curve that most clearly accords with the observed adolescent curve is shown at the right of Fig 2 in the dotted line There is assumed a substratum of generalized growth which, after 12 years of age, gradually declines from 1.9 kilos per annum to 0.5 kilos The theoretical curve is drawn in accordance with the formula

$$y = \frac{n}{\sigma \sqrt{2\pi}} e^{-1/2 \left(\frac{x}{\sigma}\right)^2}$$

where $n = 19,000$ kilo-years, $\sigma = 1.70$ years, and $y_0 = 4.47$ kilos

After subtracting the two special curves from the general curve of increments there remains a residual curve. This begins at somewhere about 9 months after birth, rises to the level of the total curve, and coincides with it during the period from 3 to 10 years. That the residual curve does not start at conception would not justify the conclusion that there is no basal growth occurring, independent of the circumnatal spurt, but indicates only that the circumnatal spurt is of such high velocity and that of basal growth is of such low velocity that the latter is quite obscured by the former. From $3\frac{1}{2}$ to 6 years growth is apparently entirely residual and it is very steady and constant, at about 1.85 kilos per annum. There is a slight rise during the 7th year of life to a new constant velocity of growth of 2.4 kilos per annum. This rate of growth continues until 11.5 years is reached at which time the adolescent spurt has already started. The basal increment now diminishes rapidly as the adolescent spurt speeds up. It remains at about the 1.225 kilo level of annual increment from 13.5 to 17.5 years of age and then falls away to the constant rate of 0.45 kilos per year which is reached at 19 years. Statistics gathered by insurance companies (Medical Actuarial Mortality Investigation (1912)) indicate that weight increases, in the male of average stature, about 1 pound a year from 20 to 26 years and then about $\frac{1}{2}$ pound yearly to 45, as stated above.

4 DISCUSSION

The early optimism as to the possibility of resolving the total growth curve of man into three "growth cycles" superimposed upon one another (Robertson (1923)²) has given way to the recognition of the great complexity of this growth curve. Thus Brody (1926)³ states that "the smoothed time curve of growth in weight [presumably of mammals, in general] is sigmoid, but the point of inflection, or rather region of inflection, is not in its center but where slightly over one-third of the mature weight is reached. The growth curve of man is the only exception encountered." Now our data show two points of inflection that clearly approximate the logistic curve. One occurs at birth and one at 15 years, in the male. Our data do not reveal the S-shaped

² Robertson (1923) p. 446.

³ Brody (1926) p. 235.

curve at "the third, fourth and fifth years" which Robertson finds (1923)⁴, and we fail to find in Robertson's or Brody's papers any sufficient evidence of this third or "juvenile" cycle (Compare my discussion of this matter (1926) ⁵)

What do the facts, as revealed in the curve of velocity (Fig 2), show clearly? Besides the circumnatal and adolescent growth cycles there is a mass of growth of irregular velocity from 2 to 10½ years, in the male, which tails away toward 20 years but continues on to 50 years of age, or later. This residual curve does not fall into any autocatalytic cycle. The existence of growth outside of "the three growth cycles" has been recently recognized by Robertson (1926)⁶ who introduces the idea and the term of "linear increment." In the mouse he conceives this to begin at about 10 weeks after birth and to increase in arithmetical fashion to 140 weeks, or later. This "linear increment" conception was forced from the fact that growth of mice "continues very slowly for long after the attainment of sexual maturity and dimensions which might readily be supposed to be 'adult' and, therefore, maximal. It is possible that in other animals also a similar linear accretion is occurring, and has escaped attention for lack of data concerning the late growth of the animals."

Now I suspect that the residual curve indicated by the dash line and, in part, by a full line in Fig 2, corresponds to Robertson's "linear accretion," inasmuch as it continues past maturity, but in detail it is entirely different from Robertson's "linear accretion" since it is not a straight line at all.

One may propose a hypothesis as to the meaning of this residual curve. One may base it on the probability that besides the natal and adolescent growth accelerators there are other growth processes of particular organs or of the body as a whole. These constitute the substratum of growth of which the natal and the adolescent cycles are especially activated or accelerated episodes. Indeed, it is plain from such studies as Riddle (1925) has made on the growth of organs in the pigeons and which Scammon (1925, 1926, *a, b*) is making on the growth of organs in man, that the total growth is, as it were,

⁴ Robertson (1923), pp 445 and 446

⁵ Davenport (1926), pp 210-212

⁶ Robertson (1926), pp 469-473

the summation of growing parts or organs, each following a more or less independent law. Not until we understand the changes in weight of the different parts of the body from the beginning of development to maturity shall we be enabled to give an adequate interpretation of the growth curve. When that happens we shall first be in a position to direct and modify the form of the developmental curve.

To illustrate, merely, the view of an important substratum of growth apart from the cycles I may refer to the findings in respect to the rate of development of three or four human organs in comparison with the growth of the body as a whole.

Thus Starkel and Wegrzynowski (1910) and E. Thomas (1911) find that the suprarenals grow rapidly in the fetus, attaining, at or about birth, a weight of 3 gm. After birth the weight falls, absolutely, to about 1.5 gm. at about 12 months of postnatal life. It then increases very slowly to about 3 gm. at about the end of 5 years. Thomas shows that the degeneration after birth affects, especially, the deeper layers of the cortex. Scammon (1926, b)⁷ shows, in addition, that in the suprarenals there is no extraordinary prenatal acceleration of growth but only a postnatal involution. A similar postnatal retardation of growth velocity occurs in the cerebellum (Scammon and Dunn (1924)).

The length of the uterus in the fetus undergoes extraordinary changes that have been worked out by Scammon (1926, a)⁸. Thus in the 7th fetal (lunar) month the uterus begins to show an extraordinary spurt in growth, as compared with the body as a whole. At birth the length of the uterus is 35 mm. while, had the spurt not occurred, it would have been only about 23 mm. Within 3 weeks after birth the length of the uterus has fallen to 24 mm, and then increases slightly during the following 5 months. "This suggests," says Scammon, "that the growth of the uterus in the latter fetal months consists of a substrate of typical fetal growth plus a secondary growth increment, which, presumably, is due to an extra stimulus furnished by a hormone of placental or possibly ovarian origin. After birth the organ loses this secondary increment but retains that result-

⁷ Scammon (1926 b), p. 809

⁸ Scammon (1926 a), p. 690

ing from the early fetal growth rate " Again, reference may be made to the well known case of the thymus, which, according to Hammar (1921),⁹ undergoes a rapid reduction of size and function as adolescence sets in at 11 to 15 years This involution seems to be determined and controlled by the development of the gonads

The foregoing interesting studies on variations in the velocity of growth of human organs justify the conclusion that the development of weight in man is the resultant of many, more or less elementary, growth processes When some special activator of development causes one or more organs simultaneously to increase in velocity of growth to a high degree then a marked maximum occurs in the human growth curve, and this may assume the form of the logistic curve of growth Two of these episodes are of overwhelming importance The great number of smaller growth operations are less outstanding, and overlap in time to such a degree as to become submerged in a nearly uniform, high and prolonged wave of growth It is probable that some of these growth impulses affect not merely one or two organs of the body but are diffused more or less uniformly throughout the entire body It is this substratum of the growth process which deserves special study and analysis in the future

Finally, one is led to speculate on the nature of the activators of the two principal special growth accelerations—the circumnatal and the adolescent There is some ground for entertaining the hypothesis that the adolescent spurt is especially activated by the secretions of the pituitary gland, or anterior lobe of the hypophysis, since preadolescent hypophyseal underactivity results in reduced growth and preadolescent hyperactivity in giant growth

The tremendous velocity of growth in the circumnatal cycle seems to be activated by something coming into the fetus from the mother through the placenta Hardly otherwise can we account for the fact that the growth process ceases its acceleration at just the time when the placental connection is broken

Experiments should throw light on the nature of the special, as well as the general, growth activators at different stages of development

⁹ Hammar (1921), p 551

5 SUMMARY OF CONCLUSIONS

The human growth curve shows two (and only two) outstanding periods of accelerated growth—the circumnatal and the adolescent

The circumnatal growth cycle attains great velocity, which reaches a maximum at the time of birth. The curve of this cycle is best fitted by a theoretical skew curve of Pearson's Type I. It has a theoretical range of 44 months and a standard deviation of 5.17 months. The modal velocity is 10.2 kilos per year.

The adolescent growth cycle has less maximum velocity and greater range in time than the circumnatal cycle. The best fitting theoretical curve is a normal frequency curve ranging over about 10 years with a standard deviation of about 21 months and a modal velocity of 4.5 kilos per year.

The two great growth accelerations are superimposed on a residual curve of growth which measures a substratum of growth out of which the accelerations arise. This probably extends from conception to 55 years, on the average. It is characterized by low velocity, averaging about 2 kilos per year from 2 to 12 years. It is interpreted as due to many growth operations coincident or closely blending in time.

Our curve shows no third marked period of acceleration at between the 3rd and 6th years.

The total growth in weight of the body is the sum of the weight of *its constituent organs*. In some cases these keep pace with the growth of the body as a whole, great accelerations of body growth are due to great accelerations in growth of the constituent organs. In other cases one of the organs of the body (like the thymus gland) may undergo a change in weight that is not in harmony with that of the body as a whole.

The development of the weight in man is the resultant of many more or less elementary growth processes. These result in two special episodes of growth and numerous smaller, blending, growth operations.

Hypotheses are suggested as to the basis of the special growth accelerations.

I take this occasion to acknowledge the valuable assistance of Miss Mary T. Scudder in the calculation of the two theoretical curves of Fig. 2.

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THE EFFECT OF ENZYME PURITY ON THE KINETICS OF TRYPTIC HYDROLYSIS

By HENRY BALDWIN MERRILL

(From the Laboratory of A. F. Gallun and Sons Company, Milwaukee)

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INTRODUCTION

In the course of an investigation of the function of pancreatic enzymes in the tannery process known as bating, the author has had occasion to study the rate of digestion of keratose by trypsin¹. This study has resulted in the accumulation of a considerable mass of data bearing on the kinetics of the reaction under different experimental conditions. Examination of these data from the point of view of the law of mass action has brought out an interesting relation between the purity of the enzyme specimen employed and the apparent order of the reaction. It appears from this work that the less refined the enzyme, the more closely does the digestion follow the course of a monomolecular reaction. The evidence for this finding, and its bearing on the mechanism of the inactivation of trypsin, form the subject matter of this paper.

Keratose—Since this material has not previously been studied as a substrate for enzyme action, a brief description is called for. Keratose (to apply a general term to what may or may not be a group of substances) is the first product of the alkaline hydrolysis of keratin. In its physical chemistry, keratose resembles casein, being soluble in dilute acid or alkali, but insoluble at its isoelectric point, which has been found to lie at pH = 4.1¹. The method used in this laboratory for preparing keratose² consists of dissolving clean calf hair in dilute sodium hydroxide, neutralizing to pH = 8.0, filtering off any undecomposed hair, precipitating keratose in the filtrate at its isoelectric point, and washing repeatedly by decantation. The white, curdy

¹ Wilson, J. A., and Merrill, H. B. *Ind. and Eng. Chem.*, 1926, xviii, 185.

² Wilson, J. A., and Merrill, H. B., *J. Am. Leather Chem. Assn.*, 1926, xxi, 2, 50.

precipitate so obtained is redissolved in dilute NaOH, and brought to pH = 8.0, which has been found to be the pH value at which tryptic hydrolysis of keratose is most rapid¹

Enzymes—The enzymes used in this work were commercial samples submitted to this laboratory for test as possible bating materials. They were used without any purification. The samples varied in strength from a U. S. P. pancreatin, the activity of which, measured on casein, was 7 Fuld-Gross units, to a purified trypsin of 333 Fuld-Gross units. Out of all the samples examined, three, representing respectively a very weak, a moderately strong, and a very strong preparation, were employed for the work covered by this paper. The characteristics of these samples were as follows:

Sample No	Activity measured on	
	Casein (Fuld Gross units)	Keratose ² (Wilson Merrill method)
9	333	133
6	83	27.3
2	7	4.2

EXPERIMENTAL

Method

The experimental method employed in studying the rate of digestion of keratose by enzymes is very similar to that used by Northrop² in his work with casein. The method is based upon the fact that keratose, like casein, is insoluble at its isoelectric point, while its products of digestion are soluble. Starting with a known quantity of keratose, the fraction remaining undecomposed at the end of any given time may be determined gravimetrically, and the quantity of keratose digested determined by difference. This method has the great advantage, as pointed out by Northrop, that only the first step of the digestion is studied.

The stock solution of keratose is analyzed by precipitating a measured volume at pH = 4.1, filtering through tared filter paper, drying at 100°C, and weighing. From the analytical results, the volume of stock solution containing exactly 2.000 gm keratose is calculated. This volume is placed in a liter flask with 100 cc of the powerful citrate-phosphate-borate buffer solution (pH = 8.0) described by Northrop, and made up nearly to 1 liter. The flask is placed in the thermostat and allowed to come to the desired temperature, then the enzyme, dissolved in a little water, is added, the solution is made up to 1 liter, and well

² Northrop, J. H., *J. Gen. Physiol.*, 1922-23, v, 264

shaken 100 cc. aliquots are removed immediately after adding the enzyme and at suitable time intervals thereafter. The undigested keratose is precipitated at $\text{pH} = 4.1$ by running each aliquot into 50 cc. of a sodium acetate-acetic acid buffer $\text{pH} \approx 3.6$, $N/2$ in acetate ion. The precipitate is allowed to settle, filtered through a tared paper, washed four times with very dilute HCl ($\text{pH} = 4.1$), dried and weighed. The difference between the initial weight of keratose and that obtained after any given time interval gives the weight of keratose digested in the interval.

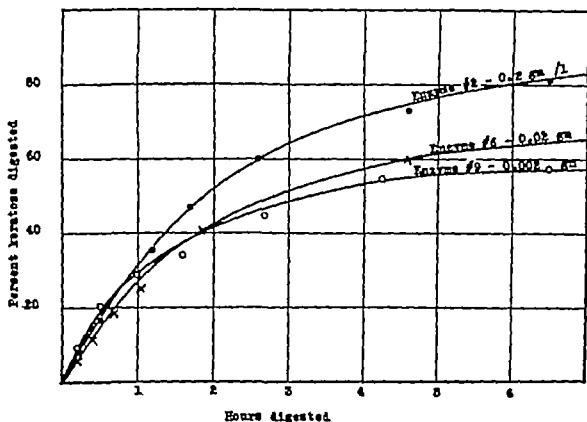


FIG. 1 Rate of digestion of keratose with different enzymes. 2.0 gm keratose per liter, $\text{pH} \approx 8.0$, $T = 35^\circ\text{C}$.

Suitable blanks were run, and corrections made for insoluble matter added with the enzyme, and for keratose digested in the absence of the enzyme. This last correction is negligible at the temperature employed for these experiments.

The temperature employed was $35^\circ\text{C} \pm 0.05^\circ$.

Tests showed that the buffer employed kept the pH value constant to within 0.1 pH unit during the course of the digestion.

Bacterial action was inhibited by the use of thymol.

Calculations—The per cent of the total keratose digested in different time periods was plotted against time for each series, and a smooth curve drawn through the points. Fig. 1 shows a set of

such curves, obtained with the three enzymes employed in this work. It is seen that most of the points lie on or close to the curves, indicating that the percentage error is small for work of this type.

The percentages digested at appropriate time periods were read off the smoothed-out curves, and employed in the calculations.

Order of Reaction—By the method outlined above, the rate of digestion of keratose was determined, using the three enzyme specimens under investigation. The quantity of enzyme added was so

TABLE I

Variations of the Velocity Constant with the Stage of the Reaction with Different Enzyme Specimens

$$k_1 = 1/t \log [a/(a-x)]$$

$a = 1$, x = fraction of a digested in t hours, temperature = 35°C

Enzyme No. 2—0.2 gm. per liter								
t	0 27	0 50	1 2	1 7	2 6			
$a - x$	0 920	0 832	0 646	0 532	0 401			
$k_1 \times 10$	(1 34)	1 60	1 58	1 61	1 53			
Enzyme No. 6—0.02 gm. per liter								
t	0 3	0 7	1 2	2 0	3 0	4 6		
$a - x$	0 910	0 810	0 720	0 610	0 510	0 400		
$k_1 \times 10$	1 36	1 31	1 19	1 07	0 97	0 86		
Enzyme No. 9—0.002 gm. per liter								
t	0 2	0 5	1 0	2 0	3 0	4 0	5 0	6 0
$a - x$	0 900	0 800	0 710	0 610	0 535	0 485	0 450	0 430
$k_1 \times 10$	2 3	1 9	1 5	1 1	0 91	0 78	0 69	0 61

adjusted that digestion took place at approximately the same rate in all three cases. The experimental data was plotted (Fig. 1), and values for the per cent keratose digested were read off at appropriate time intervals. The velocity constant of the reaction was calculated, using the equation for a monomolecular reaction

$$k_1 = 1/t \log [a/(a-x)]$$

placing $a = 1$ and x = the fraction of the total keratose digested in t hours. Briggsian logarithms were used.

The results of these calculations are presented in Table I, and are plotted in Fig 2. It will be seen that for Enzyme No 2, the weakest enzyme employed, the values obtained for k_1 are practically constant during the first 60 per cent of the reaction. In other words, the digestion does follow the course of a reaction of the first order. With No 6, which is some 7 times as strong as No 2, the values obtained for k_1 drop off rapidly, and with No 9, a preparation having 30 times

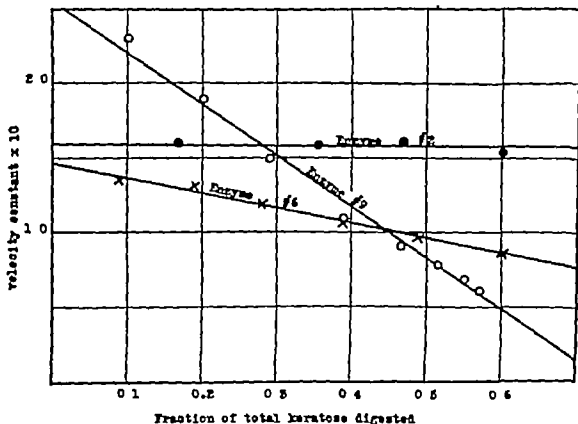


FIG 2. Variation of velocity constants with stage of reaction for different enzymes.

the activity of No 2, the rate of decrease of k_1 is very rapid,—the “constant” falling off to about one fourth of its initial value when the reaction is little more than half completed.

In Fig 2, k_1 is plotted as a function of the fraction of substrate digested. For the ideal reaction of the first order, the plot would be, obviously, a horizontal line. This condition is closely approached with Enzyme No 2. With No 6, and still more with No 9, the departure of k_1 from constancy is sufficiently apparent.

It is significant that the plots of k_1 as a function of *fraction decomposed* are straight lines. This means that k_1 decreases in value in proportion to the amount of proteose hydrolyzed, and not in proportion to the length of time elapsed since the beginning of the reaction.

Inactivation of Trypsin—The failure of an enzyme reaction to obey the law of mass action is commonly ascribed to progressive inactivation of the catalyst while the reaction is taking place. Two types of inactivation have been distinguished,—(1) a spontaneous, irreversible destruction of the enzyme that occurs in solution whether or not the enzyme acts on a substrate, and (2) a reversible "inhibition," due to combination between the enzyme and the products of the reaction. Northrop⁴ has shown that an equilibrium, governed by the law of mass action, is set up between free trypsin and "inhibitor," on the one hand, and the complex "trypsin-inhibitor" on the other. Only the free trypsin can undergo spontaneous inactivation. The author's findings may be explained on very similar grounds.

We may assume that, in the solid state, trypsin exists in combination with some inert substance. Let us further assume that this combination, which we shall designate by the formula EnIn , dissociates in solution in a manner analogous to the dissociation of a weak acid or base. The amount of active enzyme, En , existing at any time will then be fixed by the relation

$$[\text{En}] = [\text{EnIn}] / (k_d [\text{In}])$$

where k_d is the dissociation constant, and the bracketed symbols indicate concentrations.

Let us further assume that during purification of an enzyme, the concentration of active enzyme with respect to the inactive carrier is greatly increased. In a very impure preparation, $[\text{In}]$ will be large, and $[\text{En}]$ correspondingly small. The preparation will then be one of low activity. On the other hand, the undissociated complex, EnIn , will serve as a reservoir for En , more active enzyme being liberated as that present initially is used up by inactivation or combination with the products of the reaction. Thus $[\text{En}]$ will remain practically constant during the course of the digestion. These are

⁴ Northrop, J. H., *J. Gen. Physiol.*, 1921-22, 15, 227, 245, 261.

the conditions which exist when a very weak enzyme, such as No 2, is used for protein digestion, and, as we have seen, the constancy of enzyme concentration during the course of the reaction is reflected in the closeness with which the hydrolysis follows the course of a first order reaction

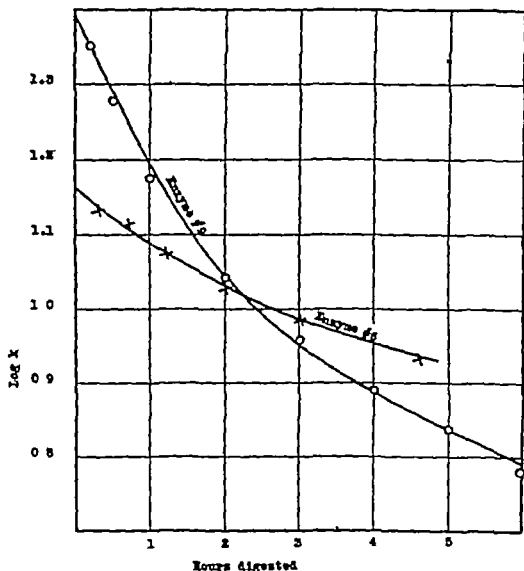


FIG. 3 $\log k$ as a function of time

In a highly purified enzyme, where the concentration of En is high with reference to the inert material, the complex $EnIn$ will be largely dissociated at the start, and the preparation will manifest high activity. The free enzyme will, however, be inactivated or inhibited rapidly, and since no sufficient reservoir of combined enzyme

exists, the concentration of active enzyme will diminish. Under such conditions, the rate of hydrolysis of the substrate must fall off more rapidly than would be predicted from the equation for a first order reaction. Such is the case with Enzyme No. 9.

The manner in which the velocity constant of the reaction falls off affords information as to the type of inactivation which the enzyme is undergoing. Northrop has shown that the spontaneous inactivation of trypsin follows the course of the monomolecular reaction. If k_1 is proportional to the quantity of active enzyme present, then (if the enzyme is decomposing spontaneously) $\log k_1$ should be proportional to t , and the plot of $\log k_1$ against t should be a straight line. In Fig. 3, $\log k_1$ has been plotted against t for the data obtained with Enzymes Nos. 6 and 9. It is plain that k_1 does not fall off according to the equation for first order reactions. In the reversible inhibition of trypsin by combination with the products of digestion, the amount of enzyme inhibited is proportional to the fraction of substrate decomposed, and therefore k_1 will be inversely proportional to x . That this is true in the author's experiments is shown by the fact that the graphs of k_1 against x are straight lines (Fig. 2). This indicates that in these experiments the spontaneous inactivation of the enzyme is negligible, and that we are dealing chiefly with the reversible inhibition due to combination with the reaction products.

It is of interest to note that if the rate of inactivation of the enzyme happens to be the same as the rate of decomposition of the substrate, the course of the main reaction will apparently be that of a bimolecular reaction. This happens to be the case with Enzyme No. 6. The following values for the bimolecular reaction velocity constant, k_2 , were calculated from the familiar formula

$$k = \{1/t\} [x/(a - x)]$$

from the data given in Fig. 1

t	0.3	0.7	1.2	2.0	3.0	4.6
$a - x$	0.91	0.81	0.72	0.61	0.51	0.40
$k_2 \times 10$	3.3	3.3	3.2	3.2	3.2	3.3

With Enzyme No. 2, the corresponding values for k_2 increase as the hydrolysis proceeds while with No. 9 they fall off, indicating in the

latter case that the enzyme is being used up at a relatively greater rate than the substrate

SUMMARY

The rates of digestion of Lactose have been determined with three commercial enzymes, ranging widely in strength. It has been found that the weaker the enzyme preparation, the more nearly does the course of the hydrolysis conform to that of a reaction of the first order. This has been explained on the assumption that in solution an equilibrium exists between active enzyme, and enzyme combined with inert material. In very impure enzyme preparations, the large quantities of combined enzyme act as a reservoir for active enzyme, maintaining a constant concentration of active enzyme during the course of the digestion.

The author wishes to acknowledge his indebtedness to Mr John Arthur Wilson for many helpful suggestions during the preparation of this paper, and for permission to publish.

TEMPERATURE AND FREQUENCY OF HEART BEAT IN THE COCKROACH

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I

It has been found that the relation existing between temperature and the frequency of rhythmic neuromuscular activity, especially when derived from many careful measurements made upon single animals at short intervals over a fairly wide range of temperature (Crozier and Federighi, 1924-25), may throw light on the question of the identity of the chemical reactions supposed to control homologous biological processes (Crozier, 1925-26, b). The present paper reports experiments performed further to test this hypothesis by determining the temperature characteristic for the frequency of the heart beat in *Blatta orientalis* L. Observations were made from large nymphs¹ of this species. All were secured in one lot from Birmingham, Alabama, early in 1926, and were kept at room temperature, with sufficient air, in a wire cage, where food (such as raw potato, butter, breadstuffs, and dead cockroaches) was available.

The data obtained possess added value in that the animals providing them remained whole and uninjured during the experiment. A 1000 watt lamp in a stereopticon lantern proved adequate to reveal clearly the pulsations of the dorsal blood vessel even in very darkly pigmented specimens, as viewed by the transmitted light with a low power binocular microscope. (The absence of wings in the nymphs was favorable to this procedure.) The animal under observation, 22-26 mm. in length, was held in a glass tube, 30 cm. long and of such diameter as to press slightly upon the lateral edges of the terga. Wire gauze plugs, surfaced with cotton and set in place by wires

¹ Since one individual of the size used assumed wings at the next moult, these were presumably in the last nymphal instar.

attached to them, served to prevent movement forward or backward in the tube. The latter was connected at one end by way of rubber tubing to a suction pump, and at the other opened indirectly to the air of the laboratory, with the enclosed cockroach it was held immersed in the water of a rectangular thermostat. The bulb of a sensitive thermometer (graduated to 0.1°C) was enclosed in air within a length of glass tubing of the same cross-section as that of the tube holding the animal, and was held close to the latter in order to approximate the same conditions. The light from the lantern, after passing through infra-red filters, was admitted to the thermostat only through an opening in a shield covering the exposed side. Here a lens concentrated the rays upon the ventral surface of the animal. A second shield of metal, fitted to the tube and with a small opening admitting illumination only to the thoracic sterna, protected the eyes of the observer from the glare and reduced photic excitation of the animal. A larger opening in the shield on the opposite side of the tube exposed to view most of the dorsum of the cockroach. Besides thus partly shielding the animal, further unnecessary photic stimulation was avoided by switching off the lamp between sets of readings.

The time for nine complete heart beats was determined with a stop-watch. As the several chambers pulsate almost synchronously in the normal cockroach, and at least at the same rate, that thoracic or (first, second, or third) abdominal segment was observed which during a given reading seemed most favorably situated. For every temperature step in a series, at least three, generally five, and occasionally up to eleven separate counts were made with a view to compensating the variations. Thus the present material represents somewhat more than 1500 stop-watch readings. In general these refer to the animal in a quiescent state, but for some sets it was virtually impossible to obtain (as was regularly attempted) complete counts without some coincident movements of body or legs. This probably did not greatly alter the average times recorded, or more in one sense than the reverse, for the consequent increase or decrease of heart rate (generally the former, but often apparently first one and then the other) seemed to be compensated in the course of two or three counts, possibly because of a fairly rapid rhythm of the heart.

obvious change in the cardiac rhythm, I largely avoided making counts while such movements were more agitated

The temperature was changed in steps of about 1° , rarely as great as 2° . After each such change a minimum of 10 minutes was allowed for adjustment before the next set of readings was taken. A portion of this interval passed while the air within the tube acquired the temperature of the thermostat, but during the last 5 minutes or more the mercury remained practically constant at the new level. The temperatures were read to 0.01° , in but a few exceptional cases did they vary as much as 0.2° from the first to the last of any single set of readings, and they are easily correct to $\pm 0.1^{\circ}$.

At each change of temperature (10 to 25 minutes before each set of readings), fresh air, passing first through nearly 1 m. of thin glass tubing in the thermostat, was drawn through the tube holding the cockroach. This procedure was a more than ample check upon disturbances which a change of oxygen or carbon dioxide tension might cause. In this connection I cite the remarkable case of Cockroach 10 which remained alive 29 days continuously in the tube. Its heart rate was approximately unchanged during more than 10 days, even with experimental exposure to 38.4° (once) and to 5° (twice); after each experimental series, air was drawn through the tube but not oftener than every 8 to 48 hours.

II.

Satisfactory series of observations were completed with six individuals.² The data are transcribed in Figs. 1, 2, 3, and 4, where the logarithm of the frequency of the heart beat has been plotted against the reciprocal of the absolute temperature. The points represent the averages of the several readings made at each temperature step in a "run". It is apparent that within narrow limits of variation all the series describe straight lines. The relation of frequency to temperature therefore fits the Arrhenius equation $K_2/K_1 = e^{(R/K)(1/T_1 - 1/T_2)}$ in which K_1 and K_2 are proportional to velocity constants at the respective absolute temperatures T_1 and T_2 , R the gas constant,

² Three other individuals were previously observed in the course of developing and testing the experimental method. They yielded data not inconsistent with those about to be discussed.

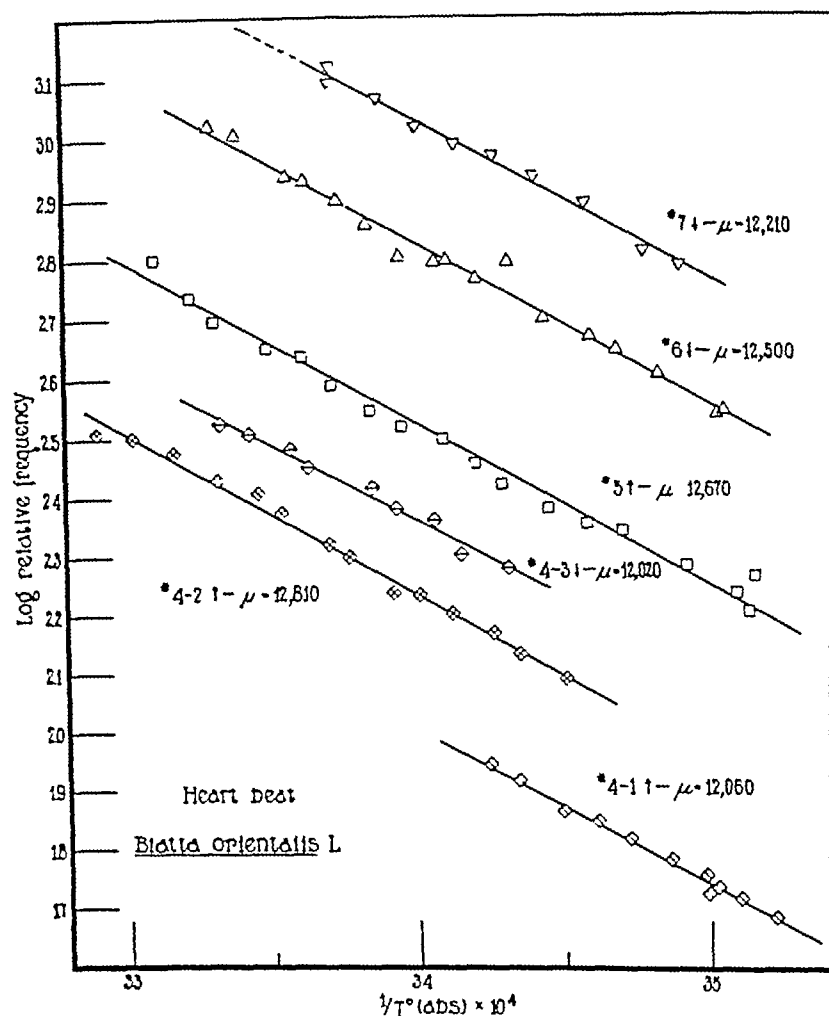


FIG 1 Series of observations with Animals 4, 5, 6, and 7. Arrows indicate whether the temperature was raised or lowered. The frequencies are factored to raise their logarithms in each of the upper five series respectively by 1.0, 0.8, 0.6, 0.4, and 0.2 above the original values. The latter do not differ between series more than about 0.1 unit at any one temperature. The size of the symbols is such as to correspond to a probable error of ± 2 per cent of each average frequency, which is greater than the probable error found by calculation. The points marked by broken line symbols in the uppermost series were obtained before the animals had become adequately accustomed to the high temperature, hence the line was fitted to the rest of this series without considering them. Such deviations illustrate the necessity for careful thermal adaptation.

and μ the critical thermal increment or temperature characteristic (Crozier, 1924-25). The value of μ is fixed by the slope of the line best fitted to the points plotted, it may be computed from the graph, the frequencies being measures of the velocity constants.

Separate and repeated fittings to the series obtained from five individuals, shown in Figs 1 and 2, lead to values of μ ranging from 12,020 to 12,810 calories for temperatures between 10° and 38°.² The weighted average $\mu = 12,500$. Of the five animals one was cooled below 10°, two series from 19° to 5° show a "break" or marked change of temperature characteristic occurring at about 10°, such that for lower temperatures μ is of the order of 18,100 (Fig. 2).

Fig. 3 is a mass plot of the series (above 10°) which are shown in Figs 1 and 2, but adjusted by suitable factors to coincide at $1/T = 0.0034$. The combined series give a straight line for which $\mu = 12,500$ calories. This represents the mean for the range 10-38°, and is subject to a probable error of ± 0.6 per cent. The vertical width of the band of points, which as in other cases (Crozier and Stier, 1924-25) forms a ribbon with parallel margins, is an index of the variability encountered in the normal cockroach. On the basis of a selection of sets of readings representing wider deviations than usual in the data here reported, the latitude of variation for any one individual is with very few exceptions well within ± 7 per cent of the mean frequency at any one temperature. On a like basis, the probable error of the mean frequencies entered in the graphs is found seldom to exceed ± 2 per cent, being much less in about half the observations.

In contrast to the others, one individual yielded data from which a clearly different magnitude of μ must be derived. When a first "run" revealed a value of the order of 14,300, I made additional series of observations upon the same individual within 18 days after the first. Considered separately, the latter series give rise to values of μ respectively higher and lower than the initial determination, yet safely

² A very slightly lower average value is given by the "runs" of falling temperatures than by those of rising temperatures, but the difference is not great or consistent enough to be significant. Thus, of six comparable series (Fig. 1) the three during which the temperature was lowered give 12,020, 12,210 and 12,500 (mean = 12,240) and the other three during which the temperature was raised give 12,060, 12,670 and 12,810 (mean = 12,510).

HEART BEAT IN THE COCKROACH

Heart beat
Blattella orientalis L

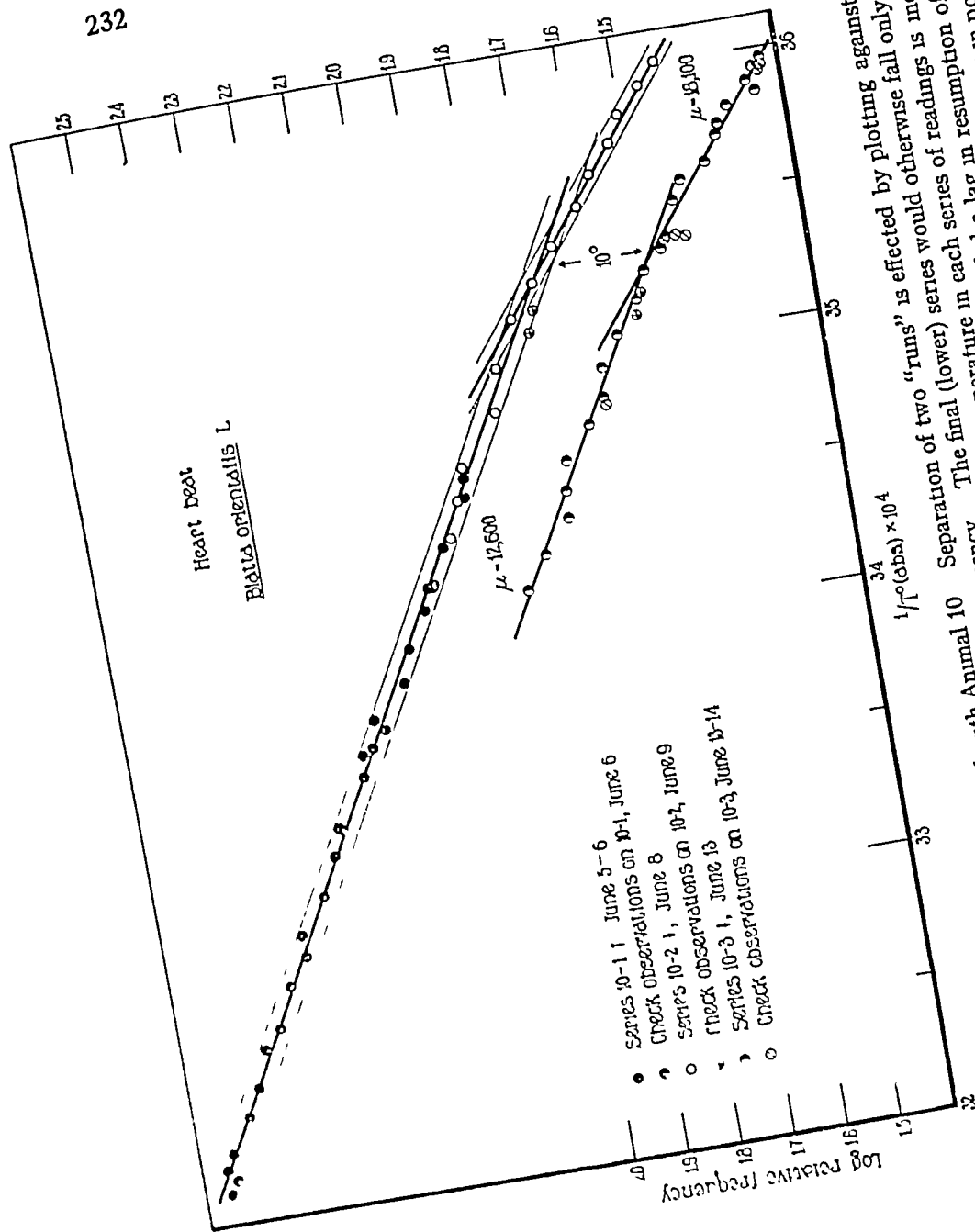


FIG 2 Series of observations obtained with Animal 10 Separation of two "runs" is effected by plotting against ordinates at left and right, the scales differing by 0.2 of log frequency The final (lower) series would otherwise fall only about 0.007 unit below the combined upper series The direction of change of temperature in each series of readings is indicated by the arrows (Since check observations on returns from extreme low temperatures revealed a lag in resumption of higher frequency, such points were not accorded full weight in fitting the lines) It is to be observed that if one were in possession merely of data from a series of readings over the temperature range 5-19°, difficulty would be experienced in obtaining a significant μ , and the data as plotted might seem to fall upon a curve

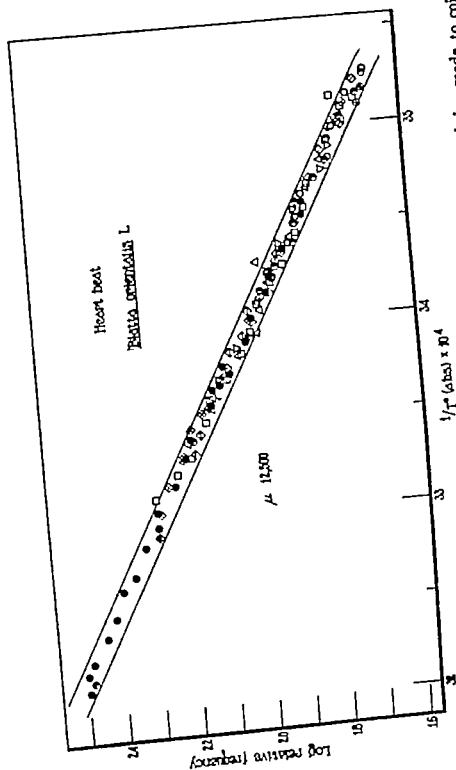


FIG. 3 Combination of series (above 10°) obtained with Animals 4, 5, 6, 7, and 10, all series being made to coincide (by suitable factors) at $1/T = 0.0034$. The symbols are the same as in Figs. 1 and 2.

HEART BEAT IN THE COCKROACH

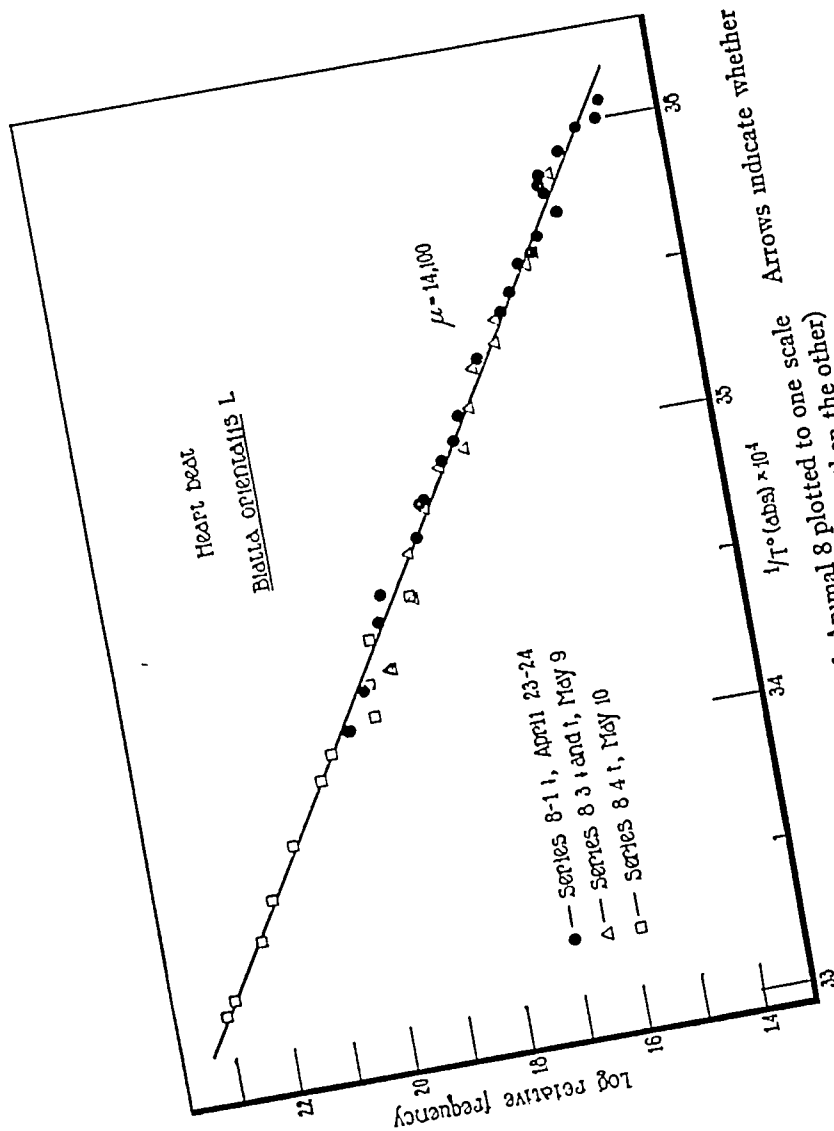


FIG 4 Three series obtained with Animal 8 plotted to one scale

comparable and not to be classed with the previous cases averaging 12,500. Fig 4 shows the data from three "runs." Coincidence is so nearly complete that a single line is drawn to represent the average, according to which $\mu = 14,100$ (correct to ± 300 or better) over the range $4.5-28^\circ$.⁴ In this case no change to a higher temperature characteristic near 10° is demonstrable for the lower range of temperature. Excepting the first series, the latitude of variation is greater than for the other five animals (as much as double), yet the probable errors of the individual plotted frequencies only rarely exceed ± 2.5 per cent. For this animal, therefore, both a difference in μ and the absence of a critical temperature at $10^\circ \pm$ serve to characterize the control of its heart rhythm as different from that of the others.

III.

The results make it clear that the heart rate in the late nymphal stage of the cockroach typically varies with the temperature in a way defined by $\mu = 12,500 \pm$ calories, but that a considerably higher critical increment (*ca* 18,100) probably holds for the same animals at low temperatures, the critical point (Crozier, 1924-25, 1925-26, *a*) at which the change occurs being $10^\circ \pm$. Although most of the animals reveal above this temperature an approach to the mean value 12,500, with a constancy indicated by a standard deviation amounting only to 2.3 per cent, an exceptional individual may be found which in some (still unknown) respect differs from the typical so that the control of the heart rhythm lies in a process whose μ is definitely of another magnitude, namely $14,100 \pm$.

The pulsation of the dorsal vessel of insects is considered to be controlled by the central nervous system (Zawarzin, 1910-11), and Carlson (1905-06) reported evidence of both augmentary and inhibi-

⁴ Another series obtained from this animal showed such abnormally high variability that μ could not be determined with any reasonable degree of accuracy. Except that the observations were in this instance begun immediately after transference to the tube, little cause can be given for this instability, but a correlation with it of a general drop in heart rate may be noted. Both occur in cases of sub-normal vitality as other observations show. Thus Cockroach 10 showed both a marked increase in latitude of variation and an absolute slowing down of the heart rhythm after 4 weeks in the experimental tube.

tory innervation of the heart of an orthopteran (*Dictyophorus*)⁵ The typical value of μ (12,500) for the cockroach heart rate was therefore expected to accord quantitatively with values determined for other non-respiratory neuromuscular activities of arthropods presumed to depend upon the rate of "central nervous discharge" (Crozier, 1924-25) It does so within limits of difference ascribable to errors of curve fitting or to consequences of uncontrolled body or limb movements Thus Crozier and Stier (1924-25) have listed a number of such phenomena, for which $\mu = 12,200 \pm$ More recently they have (1925-26) reported the same value applying to locomotor activity in tent caterpillars In addition, Federighi⁶ finds the heart beat of the annelid *Nereis* to show $\mu = 12,400$

Neither is the exceptional case where $\mu = 14,100 \pm$ entirely without counterpart, although it is very infrequent (Crozier, 1925-26, b) A like value appeared (as an exception in series yielding 16,200) among Glaser's (1925-26) determinations for the heart rate of a pteropod and (as exception to 11,100) in Cole's (1925-26) findings for locomotion in *Planaria* The data in the present instance offer nothing toward explaining the atypical value, for no difference was apparent in the treatment or condition of the animals All that can be said is that the results point to the possible validity of $14,000 +$ as the temperature characteristic of one of several chemical reactions which may be supposed necessary to more than one type of vital process (Crozier, 1925-26 a, b) but which only exceptionally proceeds so slowly as to assume a governing rôle

IV

SUMMARY

The frequency of pulsation of the intact heart in nymphs (final (?) instar) of *Blatta orientalis* L. increases with the temperature according to the equation of Arrhenius The constant μ has typically the same value, within reasonable limits of error, as that (12,200) deduced for other, homologous activities of arthropods where the

⁵More recently Alexandrovicz (1926) has described in detail the innervation of the cockroach heart

⁶Federighi, H. (unpublished experiments)

rate of central nervous discharge is perhaps the controlling element, namely $12,500 \pm$ calories for temperatures $10-38^{\circ}\text{C}$. Below a critical temperature of about 10° a change to a higher value of the temperature characteristic occurs, such that $\mu = 18,100 \pm$. Exceptionally (one individual) $\mu = 14,100 \pm$ over the whole range of observed temperature ($4.5-28^{\circ}$)

The quantitative correspondence of μ for frequency of heart beat in different arthropods adds weight to the conception that this constant may be employed for the recognition of controlling processes.

It is a pleasure to acknowledge my indebtedness to Professor W. J. Crozier for his suggestion and guidance of this work.

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SOME PHYSICOCHEMICAL PROPERTIES OF DISSOCIATED SPONGE CELLS

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I. INTRODUCTION

It has been known since Wilson's discovery (1) that dissociated cells of *Microciona* come together and form aggregates, which by further transformation develop into new sponges. Similar processes were observed in fresh water and in calcareous sponges (2, 3), and in hydroids and alcyonarians (4, 5). Recently one of us (6, 7), in studying the behavior of dissociated cells of *Microciona*, found that the formation of aggregates is due to the amoeboid movement of so called archaeocytes, that is, unspecialized cells of the sponge mesenchyme, which upon separation creep in various directions and coalesce with other cells of the same species which happen to lie on their route. According to these observations, aggregation is easily affected by changes in the surrounding medium. In pure isotonic solutions of NaCl or KCl the amoeboid movement is entirely inhibited, and the addition of at least one of the alkaline earth metals, either Ca or Mg, is necessary to produce the aggregation of cells. The addition of acids or bases to a suspension of cells also causes significant changes in their behavior, inhibiting their movement and changing the adhesive properties of the protoplasm. In mixed suspension, the cells coalesce only with cells of their own species, forming separate aggregates, while in alkaline sea water the *Microciona* aggregates become surrounded by the *Cliona* cells.

The present investigation is an attempt to deal in a quantitative manner with the equilibrium relations involved between the cells of two siliceous sponges, *Microciona prolifera* Ver. and *Cliona celata* Gr., and acid or base.

As a preliminary to the account of the investigation of the sponge cells, we report a titration of 0.00280 molar NaAc in 0.520 molar NaCl solution, this solution served as the medium in experiments with the cells

II The Hydrogen Ion Activity in a Solution Containing 0.00280 Mols of NaAc, 0.520 mols of NaCl per Liter, and Various Amounts of HCl and NaOH

A medium for the titration of the cells of the sponges must answer several requirements. First of all, it must be isotonic with the cells, secondly, it must be of such a nature as to prevent aggregation of the cells (7), and, thirdly, it must have a certain buffer value on the acid side of neutrality (since most of the observations were made in that range) so as to yield reproducible E M F measurements.

After several trials we found that a solution containing 0.00280 mols of NaAc and 0.520 mols of NaCl, and varied amounts of HCl and NaOH, answered practically all of our requirements. It gives fairly reproducible E M F measurements, but its buffer value is not large enough to mask the effect of the acid or base bound by the sponge cells.

Several investigations were made on acetate buffers containing different amounts of NaCl. L. Michaelis and R. Krüger (8) studied the hydrogen ion activity of a 0.02 N mixture of equal amounts of NaAc and HAc in the different salts. They found that in 1 molar NaCl the mixture has a pH of 4.484, the pK' evidently being equal to 4.484.

L. Michaelis and K. Kakinuma (9) in their contribution to the electrochemical measurements of the activity of ions found that 0.01 molar solutions of equal amounts of NaAc and HAc, containing different amounts of NaCl, have different hydrogen ion activities. A solution containing 0.1 mol of NaCl had a pH equal to 4.607, a 0.5 molar solution a pH of 4.503, and a 1.0 molar solution a pH of 4.448.

G. S. Walpole (10) investigated the pH of a mixture of HAc, NaAc, and NaCl. The concentration of Ac in this system was 0.20 N, a 1:1 mixture gave a pH of 4.58. The measurements were made at 18°C.

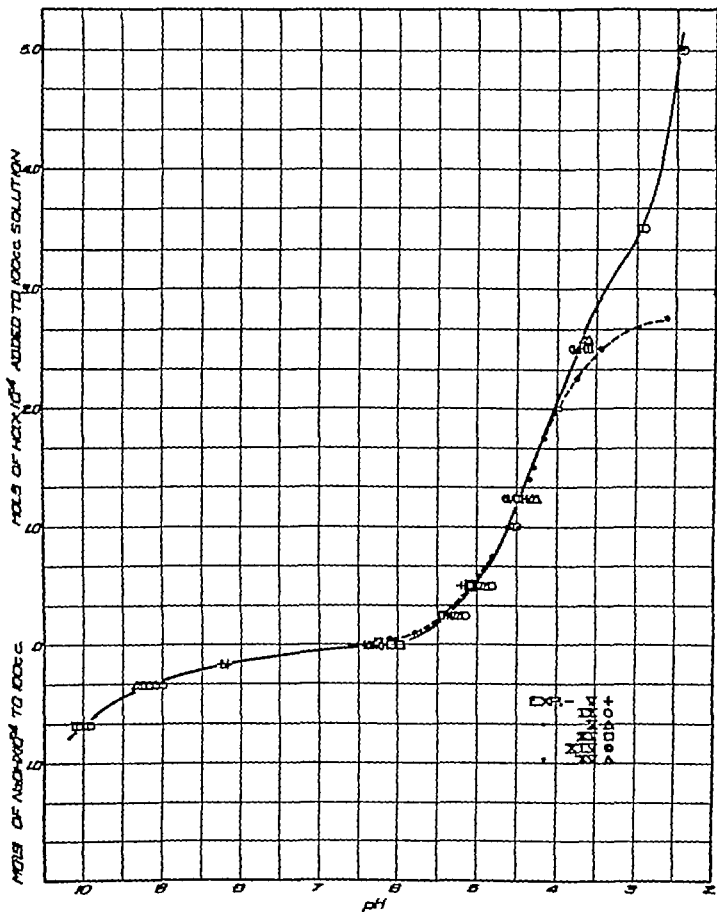


FIG. 1 The hydrogen ion activity in a solution containing 0.00280 mols NaAc 0.520 mols NaCl per liter and varied amounts of HCl or NaOH.

J N Bronsted (11) recalculated the experimental data obtained by Walpole in terms of fundamental thermodynamic functions

The results of our investigation are graphically represented in Fig 1

All the pH measurements reported were made by means of a Leeds and Northrup potentiometer The E M F of the hydrogen electrode was measured against a 0.1 N KCl calomel electrode, using a saturated KCl bridge The pH's reported were recalculated by the equation

$$\text{pH} = (\text{E M F observed} - \text{E M F calomel}) / 0.001983$$

For the E M F of the calomel electrode we used the value given by Lewis and Randall (12) No correction for the diffusion potential was made

Our experiments were carried out at slightly different temperatures The effect of temperature on the activity is not a negligible one We corrected for the influence of temperature by interpolating between experimental points Fig 1 represents the titration of our acetate buffer at about 21–22°C

In this figure the dotted line represents the pH values calculated by the Henderson-Hasselbalch equation

$$\text{pH} = \text{pK}' + \log [(\text{NaAc}) / (\text{HAc})]$$

It is evident from Fig 1 that the equation holds over a considerable range It fails, however, to describe the experimental data in the range where the amount of NaAc becomes very small The average pK' for acetic acid in our system is about 4.37

III The Hydrogen Ion Activity in a Suspension of Cells of Microciona prolifera or Chona celata, in Which the Amount of Acid or Base is Varied, but the Concentration of Cells Is Kept Constant

In its natural habitat *Microciona* is usually found attached to rocks or shells, frequently occurring on oyster beds The sulfur sponge, *Chona*, is a boring sponge, it infests the shells of various pelecypods (both living and dead), and having bored through them, grows farther reaching an enormous size

The sponges used in the experiments were collected in the vicinity

of the Woods Hole laboratory. *Microciona* was taken from the mouth of Wareham Bay, and from Waquoit Pond near Falmouth, Massachusetts. *Cliona* was obtained in Squeteague Harbor near North Falmouth, Massachusetts. Both species inhabit shallow waters and normally sustain considerable fluctuations in salinity. In Waquoit Pond and in Wareham River the salinity, according to our observations, varies with each tide from 27 gm per liter at high tide to 16 at low tide.

Though the salinity in the laboratory tanks at Woods Hole is much higher, varying from 31 to 32, no harmful effect was noticed, and the sponges sustained the new environment very well. As a rule, however, the sponges used for experimentation were not kept longer in the aquarium tanks than 5 days. Experience shows that as a result of prolonged life in the aquarium they undergo physiological and anatomical changes, and become unfit for experimental work.

The following procedure was adopted to obtain a suspension of sponge cells.

(1) Each piece of sponge was washed, all dead portions were cut away, and it was cleaned from all foreign substances, such as small pebbles, sand, mud, or algae.

(2) The sponge was then placed for 15 minutes in a 0.520 molar NaCl solution, the solution being changed twice.

(3) The material was squeezed through bolting silk No. 20 into a solution containing 0.00280 mols NaAc and 0.520 mols NaCl per liter.

(4) The next procedure consisted in centrifuging and washing twice with a solution of the same concentration of salts as that described in (3).

(5) The suspension of cells thus obtained was transferred to a vessel through which a steady current of CO₂-free air was bubbled. This last process was necessary in order to free the solution from any small amounts of bound or free carbon dioxide which might possibly be present, and also to keep the cells from settling to the bottom of the vessel. By bubbling air through the suspension, it can be kept for 24 hours without sedimentation and aggregation of cells.

The suspension of sponge cells obtained consists of archaeocytes, collencytes, pinacocytes, desmacytes, and choanocytes. The percentage composition of the suspension may be given as follows. *Micro-*

ciona, 25.5 per cent archæocytes, 9.9 per cent collencytes, and 64.6 per cent desmacytes, pinacocytes, and choanocytes, *Chona*, 15.4 per cent archæocytes, 15.0 per cent collencytes, 69.0 per cent desmacytes, pinacocytes, and choanocytes

During the preparation of the suspension the cells were subjected to rather vigorous mechanical treatment. Part of them might have been cytolized. The products of this cytolysis might appear in the watery phase, and might be responsible for the binding of any acid or base added to the system.

TABLE I

Effect of the Number of Washings on the pH of a Suspension of Cells of Microciona prolifera

Experiment VI. Washing solution contains 0.520 mols of NaCl, 0.00280 mols of NaAc, and 1.25×10^{-3} mols of HCl per liter. 30 minute intervals between consecutive washings.

No. of washings. (1)	E.M.F. (2)	Temperature. (3)	pH (4)
	volts	°C	
2	0.6230	23.8	4.88
2	0.6218	23.8	4.86
3	0.6274	23.8	4.95
3	0.6272	23.8	4.95
4	0.6251	24.2	4.91
4	0.6247	24.3	4.90
5	0.6235	24.2	4.88
5	0.6233	24.2	4.88
6	0.6205	24.1	4.83
6	0.6206	24.1	4.83
NaCl solution used in this experiment.	0.5952	24.7	4.40
	0.5960	24.7	4.41

In order to determine whether we were dealing in our experiments with the acid- or base-binding property of the cells, or with the effect of some unknown product of cytolysis, we carried out the experiment reported in Table I. In this experiment a suspension, prepared in the way already described, was further washed with a solution containing 0.00280 mols of NaAc and 0.520 mols of NaCl per liter. After each washing the suspension was centrifuged, and the pH of the super-

natant liquid determined. It is evident that the pH of the suspension remains practically constant. If the acid binding property of this system was dependent upon the product of cytolysis, the pH should gradually have risen until it reached the pH of the washing solution. No such phenomenon was observed. We must conclude, therefore, that in this case we are dealing with a property very closely associated with the living cells.

In our experiment we added acid or base to the suspension of the sponge cells. When any acid or base is added to a system containing

TABLE II

Effect of Time upon the Establishment of an Equilibrium between the Cells and the Acid Added

Solution 0.520 mols NaCl and 0.00280 mols NaAc per liter

Suspension 51.9×10^6 cells of *Microcystis* suspended in 100 cc. of solution
 1.25×10^{-4} mols HCl added to it.

Time elapsing between the addition of the acid and the E.M.F. measurement.	E.M.F.	Temperature.	pH
(1)	(2)	(3)	(4)
<i>min.</i>	<i>volts</i>	<i>°C</i>	
48	0.6219	21.8	4.892
	0.6218	21.8	4.890
63	0.6223	22.0	4.896
	0.6223	22.0	4.896
99	0.6241	22.0	4.919
	0.6240	22.0	4.918
125	0.6249	22.4	4.933
	0.6248	22.4	4.931

basic or acid radicals, a displacement of the equilibrium occurs. The establishment of the new equilibrium takes a certain length of time, depending upon the properties of the system. To test the effect of time on the system containing a rather large amount of acid and a suspension of cells of *Microcystis prolifera*, we carried out the experiment reported in Table II. As is seen from the table, the pH values are almost constant. The difference in the pH value of the cell suspen-

sion and the same solution without the cells, in this experiment, is equal to 0.5 of a pH unit, while the difference between the first reading and the last is only 0.04 of a pH unit. There are three possible

TABLE III
Vitality Tests of the Cells Used in the Titration Experiments
Microciona prolifera

Experiment No. (1)	pH of the suspension (2)	Condition of cells after titration experiments.	
		Immediately (3)	12 hrs later (4)
VIII	4.56	Normal	Very small aggregates slightly adhering to glass, many cytolized cells
V	4.82	"	Small globular aggregates adhering to glass, few cytolized cells
III	5.90	"	Normal aggregates adhering to glass, few cytolized cells
V	6.13	"	Normal aggregates adhering to glass
X	6.36	"	" " " "
X	6.96	"	" " " "

TABLE IV
Vitality Tests of the Cells Used in the Titration Experiments
Cliona celata

Experiment No. (1)	pH of the suspension. (2)	Condition of cells after titration experiments	
		Immediately (3)	12 hrs later (4)
IX	3.06	Part of cells cytolized	Cells strongly adhering to glass, no aggregation
IX	3.71	Normal	" " " " " "
IX	4.09	"	" " " " " "
IX	6.59	"	Normal aggregates
IX	6.70	"	" "
XI	7.34	"	" "

causes of this variation. One of them has already been pointed out, namely, the time factor in the establishment of the new equilibrium. The second factor which must undoubtedly be present in any system containing living material is that of metabolism. The products of

the metabolism might possess acid or basic properties of their own, and might gradually change the pH of the medium. Without knowing the chemical nature of these metabolic products, one cannot determine their influence upon the pH of the suspension. The third factor which may be responsible for this variation is the beginning of cytolysis. It is quite probable that in such acid solution irreversible changes occur in the cells, producing more and more titratable material. This will be seen from the discussion of the vitality tests of the cells treated with acid.

In all subsequent measurements we used 45 minutes for the equilibration time.

The next problem with which an investigator of living matter is confronted, is to determine whether or not his chemical manipulations have caused a permanent injury or death to the object of his experiment. The best test of this, is to examine the cells immediately after the experiment and to observe their behavior when they are brought back to a normal environment.

For this purpose we conducted the following tests in conjunction with the measurements of the pH which resulted from the addition of acid or base to the suspension of sponge cells. The cells used in the experiments were washed with sea water and examined under the microscope to determine whether they were alive or not, then 1 cc. of suspension was added to 9 cc. of sea water, and the mixture left undisturbed for 12 hours. This period is long enough for uninjured cells to coalesce and form globular aggregates which adhere strongly to the glass (7). The cells irreversibly affected by previous treatment are partially cytolized and form aggregates of irregular shape. Dead cells form loose sediment not adhering to the glass.

Tables III and IV list the results of the vitality tests carried out on cells taken from the titration experiments. It can easily be seen that the susceptibility of *Microciona prolifera* to acid solutions used in the experiments is much higher than that of *Cliona celata*. Though at the end of Experiments V and VIII the cells of the former remain alive and under the microscope appear to be normal, their ameboid movement is retarded. After 12 hours, instead of forming a few large aggregates as always happens under normal conditions, they coalesce into numerous small groups, many cells at the end of this period be-

come cytolyzed. This occurs when the pH of the supernatant liquid of the suspension is 4.56.

The cells of *Chona celata* endure much higher acidity remaining uninjured at pH 3.71, though their ameboid movement after such treatment is inhibited.

The difference between the cells of the two sponges can undoubtedly be correlated with the fact that for a given change in pH in fairly acid solutions, *Microciona* binds more acid than *Chona*. We may suspect therefore that greater chemical changes occur in the first than in the second.

It must be borne in mind that, due to the rough treatment during squeezing, washing, and centrifuging, the cell suspensions may contain a certain amount of cytolyzed material, so the presence of a small number of cytolyzed cells cannot be attributed entirely to the effect of acid solution. Further increase in hydrogen ion activity will certainly cause a complete cytolysis and death of the cells. As can be concluded from the examination of Tables III and IV, the critical concentration probably lies just below pH 4.5 for *Microciona* and about 3.7 for *Chona*. Above these values, the largest part of the cells examined under the microscope immediately after the titration experiments showed no evidence of injury.

The amount of acid or base bound in titration of a suspension depends upon the concentration of cells in that suspension. Our titration experiments were carried out with suspensions of a definite concentration. We shall express this concentration in terms of the number of cells present in 100 cc. of the suspension. This method of expressing the concentration is not strictly correct. Cells have their own volume, therefore the volume of "free" solution depends upon the number of cells present. Any computation of acid or base bound referred to 100 cc. will deviate from the true value by the volume of cells present in the system. However, we believe that by using rather dilute suspensions of cells we made this error negligibly small.

In determining the number of cells in a given suspension, the following procedure was adopted. 1 cc. of this suspension was diluted one hundred times and shaken well. 1 cc. of this suspension was transferred to a counting cell. A uniform distribution of the cells was secured by the use of a pipette. In about 10 minutes the cells settled

on the bottom and could be counted with a Whipple square micrometer. We counted the cells in ten fields of view taken at random at various parts of the counting cell. From the average number obtained by this procedure the total number of cells in 1.00 cc. of suspension was estimated. The results were usually accurate within 5 per cent.

If the stock suspension was found to differ from the desired concentration, it was diluted to the appropriate extent. After the dilution, the number of cells was checked once more.

In all our experiments we titrated with HCl and NaOH, and for this reason our medium contained a large amount of NaCl. Any addition of small quantities of Cl or Na produced, therefore, a practically negligible change in the concentration of either Na or Cl. Any reaction of the cells will, therefore, be due entirely to the change in the concentration of free acid or base as measured by the hydrogen ion activity or its dependent variable, the hydroxyl ion activity. This statement would be accurate if our systems had not contained NaAc. Upon the addition of an acid, however, the concentration of Ac^- decreases proportionally to the acid added. Therefore, in addition to the variables (H^+) and (OH^-) we have the variables (HAc) and (Ac^-). Evidently this second set of variables cannot be neglected. A simple way to test the influence, if any, of the concentration of HAc and Ac^- is to titrate the cells in a solution of 0.520 molar NaCl in the absence of NaAc. Such an experiment is hardly quantitative in a slightly acid solution, but in a medium containing a rather large amount of acid the E.M.F. becomes reliable. Therefore we brought a solution containing 0.520 mols of NaCl per liter to a pH of 4.50 by adding to it a known amount of HCl. Then to the same solution we added about 50×10^8 cells of *Micrococcina prolifera* and by the addition of HCl brought it to the same pH as the solution of NaCl. It was found necessary to add more acid to the cells than to the NaCl solution in order to make the two solutions isohydronic. Evidently the amount of acid added to the cells minus the amount of acid added to the NaCl solution is the amount of acid bound by the cells. It was found to be equal to 1.2 ± 0.2 mols $\text{HCl} \times 10^{-4}$. If we compare this figure with the one obtained from the titration of the cells of *Micrococcina prolifera* in the presence of NaAc (Fig. 2) we find a complete agreement. Evidently the (HAc) and (Ac^-) are not the controlling factors in the titration in question.

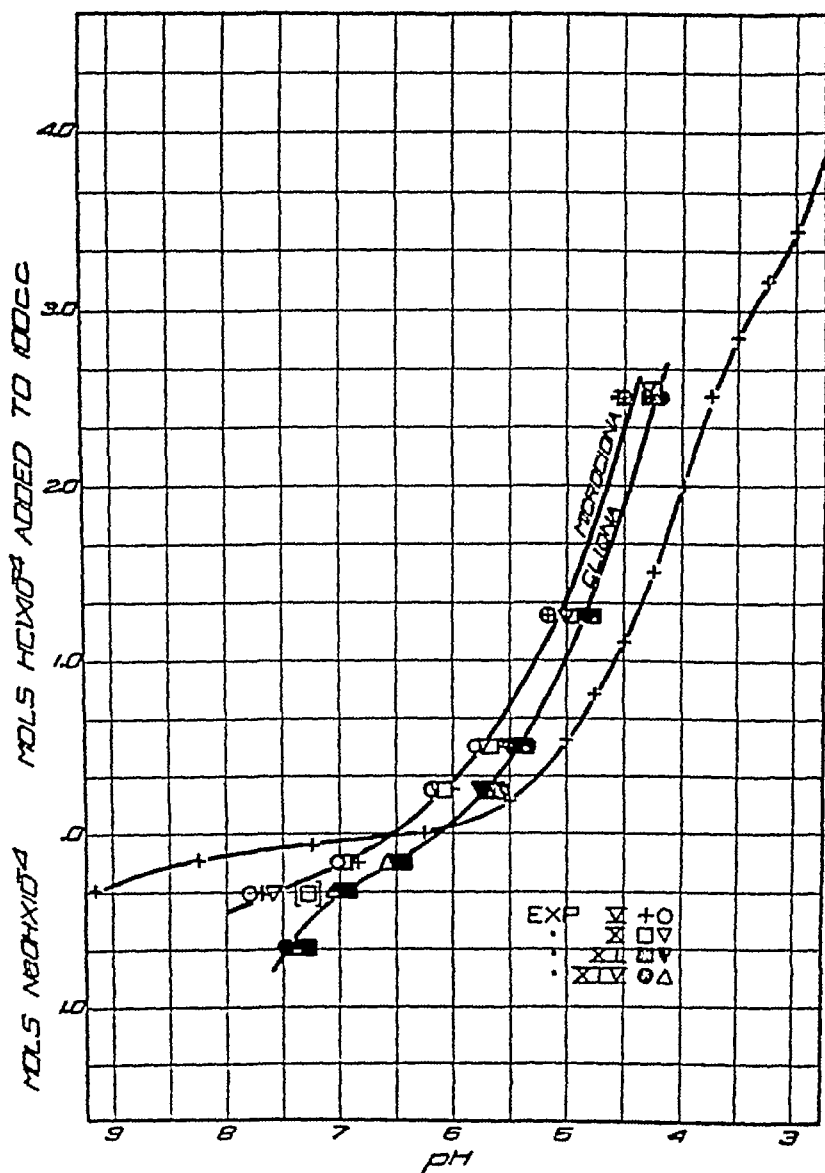


FIG 2 The hydrogen ion activity in a solution containing NaAc and NaCl, a given number of cells of *Microciona prolifera* or *Chiona celata*, and varied amounts of HCl or NaOH.

Microciona Experiment V, 52.4×10^8 cells, Experiment X, 49.3×10^8 cells
Chiona Experiment XI, 57×10^8 cells, Experiment XIV, 51.1×10^8 cells

The titration of the suspension was effected by adding to a known amount of cells a given amount of HCl or NaOH. The cells were then centrifuged and the pH determined electrometrically on the supernatant liquid. Two E.M.F. measurements were carried out on each sample. The results of two experiments are given in Fig. 2, together with the titration of the acetate buffer, taken from Fig. 1.

As may be noticed from the titration curve of *Micrococcina prolifera*, the two experiments disagree slightly with each other in the upper part of the curve. The reason for this disagreement is a difference in concentration of the cells in the two experiments. By drawing a line between the experimental points we can take care of this influence, and the line of titration would represent a titration of suspension having approximately 50.8×10^8 cells per 100 cc. A similar behavior is shown by the cells of *Cliona*, though to a lesser extent.

The titration curves obtained for the sponge cells are only functions related to the acid- or base-binding properties of the cells. The curves will have different shapes in media containing different buffers.

If we subtract at any pH the amount of acid necessary to bring the acetate buffer to that pH from the amount of acid added to the cells to bring them to the same pH, we shall obtain a value characteristic of the suspension—the amount bound by the suspension.¹

Such a calculation was made for both *Micrococcina* and *Cliona* for the slightly acid and basic ranges of the titration curve. The results of the estimates are given in Fig. 3. They are probably accurate within about 8 per cent.

The function thus obtained is of fundamental importance for the estimation of the physicochemical properties of cells. Each curve has

¹ This procedure is not strictly correct since the free base or acid is related to the hydrogen ion activity by the equations

$$(\text{HCl}) = \gamma_1 (a_{\text{H}})$$

$$(\text{NaOH}) = \gamma_2 K_w + (a_{\text{H}})$$

in which the activity coefficients γ_1 and γ_2 vary with the change in concentration of HCl and NaOH.

But, since our system contains a large amount of NaCl the change in the activity coefficients between the acetate titration curve and the one of the sponge cells is probably small.

two parts, one above the zero point where the sponge behaves as a base, and one below where it behaves as an acid. The zero point,

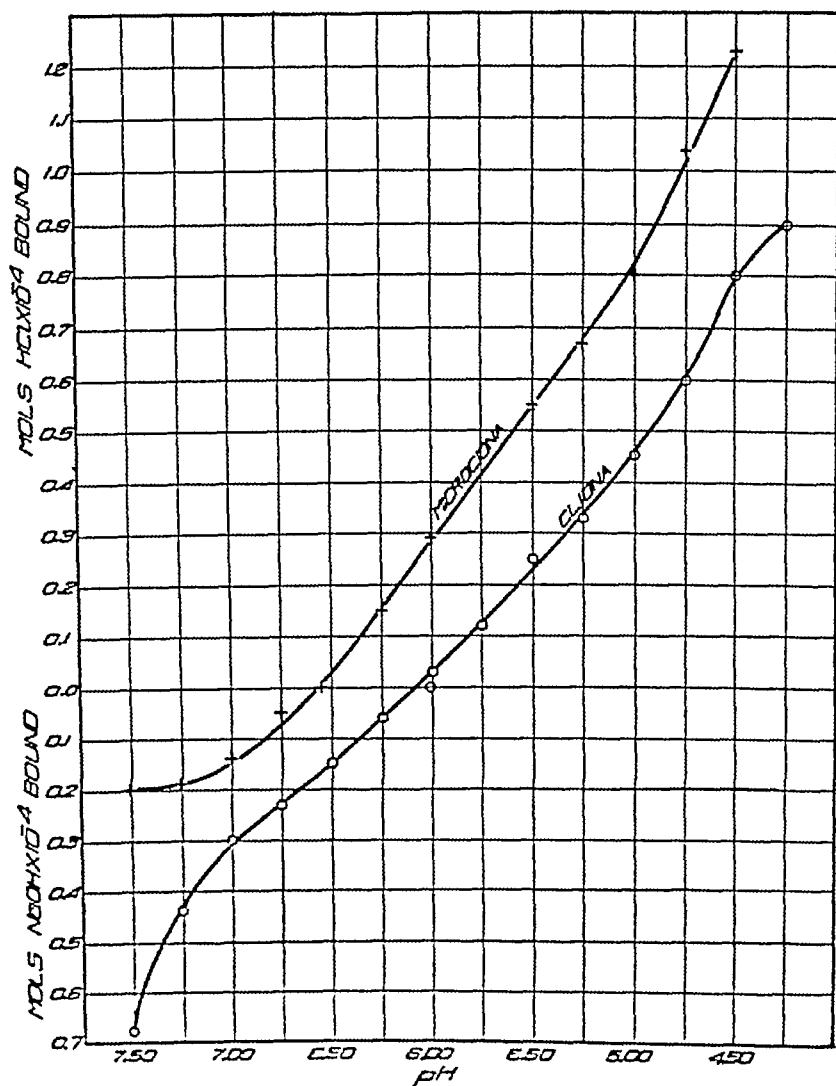


FIG. 3. The acid and base bound by the cells of *Microciona prolifera* and *Cliona* cells at different hydrogen ion activities.

where no base or acid is bound is of considerable interest for us. It represents the pH of a pure suspension of cells, extracted from the

sponge and washed with isotonic NaCl. These pH values for extracted cells are different for *Microciona* and *Cliona*. The cells of *Cliona* are more acidic than those of *Microciona*. We believe that these pH values may be characteristic of the species concerned, provided the comparison is made at a concentration of about $50-60 \times 10^4$ cells.

The hydrogen ion activity of the original suspension for *Microciona prolifera* is equal to a pH value of 6.55 ± 0.1 , and for *Cliona celata* to a pH value of 6.10 ± 0.1 .

Passing to the acid range of the acid or base-binding curve we observe that *Cliona*, being more acidic than *Microciona*, behaves as a weaker base, and binds less acid than the latter. *Microciona*, being more basic, binds more acid for a given change in pH.

The basic part of the acid or base-binding curve is even more characteristic for the two species of sponges. While *Microciona prolifera* is almost saturated with the small amount of base at a pH value of 7.50, *Cliona* still has a considerable base-binding capacity at that point. It substantiates once more the conclusion reached upon comparison of the two species in the acid portion of the curve, namely, that *Cliona* behaves as a much stronger acid than *Microciona*.

This conclusion, however, is open to one criticism: the suspensions of the cells of *Microciona* and *Cliona*, though containing an equal number of cells, are composed of cells of different sizes. Therefore, the total surface of the cells is different for *Microciona* and *Cliona*.

If the removal of acid or base from the liquid phase, by the cells, is entirely due to the effect of the active surface, the results reached in this investigation would seem to refer to the surface, but not to the chemical properties of the body of the cells.

It is therefore of considerable interest to provide an experimental evidence to prove that the reagents used penetrated inside the cells.

In the course of the investigation upon the cells of *Cliona* it was found that these cells changed their coloration from yellow to dark brown at a pH ranging from 7.3 to 7.4. Upon microscopic examination of the cells, it was observed that the yellow pigmented granules of the cells were responsible for this change in color.

On treating the cells with absolute ethyl alcohol, this pigment can be extracted, and the same change in color reproduced in a test tube.

These experiments indicate that the cells of *Cliona* in faintly alkali-

line solution are permeable to our reagents. The reaction is not limited to the surface of the cell.

This evidence cannot, however, be extended to the acid range of titration of *Chona*, nor to *Microciona* suspensions, but, since we have no reasons for believing that an entirely different physicochemical mechanism is involved in these cases, we are inclined to think that the action of our reagents is not limited to the surfaces of the cells.

We may conclude, therefore, that the concentration of cells being equal, the suspensions of cells of *Microciona* and *Chona* differ from each other in their physicochemical properties, the comparison being made on suspensions of specified composition.

IV CONCLUSIONS

1 The activity of the hydrogen ion, in a system containing 0.00280 mols of NaAc, 0.520 mols of NaCl per liter, and varied amounts of HCl or NaOH has been investigated. The average value of pK' for acetic acid in this system is about 4.37.

2 The effect of the addition of various amounts of HCl and NaOH to a system containing 0.00280 mols of NaAc, 0.520 mols of NaCl, and a known number of cells of either *Microciona prolifera* or *Chona celata* was then studied. It was found that in weak acid solutions *Microciona* behaves as a stronger base than *Chona*, the former being practically saturated with base at a pH of 7.5. Similar behavior is shown by suspensions of cells to which no acid or base was added: the cells of *Chona* are more acidic than the cells of *Microciona*.

3 The microscopic examinations of the cells subjected to the treatment with acid or base indicate that the cells of *Microciona* remain alive down to pH 4.50, the cells of *Chona* sustain greater acidity,—at pH 3.7 they exhibit no signs of cytolysis. Tests for aggregation of these cells showed that this phenomenon is greatly inhibited even by slightly acid solutions.

4 The conclusion is drawn that the concentration of cells being equal, the suspensions of cells of *Microciona* and *Chona* differ from each other in their physicochemical properties, the comparison being made on suspensions of specified composition.

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THE GEOTROPIC CONDUCT OF YOUNG RATS

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I.

If tropistic behavior is to be utilized for ultimate analysis of the inner processes controlling conduct it is quite necessary that the most complete possible mathematical expressions be found for at least several different modes of response. Only in the case of phototropism has any considerable progress in this direction been achieved. We have reference, not so much to the theory of sensory activation, as to the reasonably complete formulation of relationships between the magnitude of the excitatory intensity and the speed and extent of the induced orientation. In this respect the knowledge of geotropism, by contrast, is singularly defective.

For plants, it is found that geotropic excitation is proportional to the sine of the angle of the stimulated part with the horizontal (Fitting, 1905, Pekelharing, 1910). From the relationship between mass of attached leaf and rate of geotropic curvature in horizontal stems of *Bryophyllum*, Loeb (1918, 1924) inferred that the curvature was dependent upon the amount of (gravitationally directed) substance sent into the stem by the leaf. This is obviously consistent with the finding that the "presentation time" for geotropic response is directly proportional to the effective gravitational component (Pekelharing, 1910).

Quite recently the question of geotropic orientation in animals has been reexamined by Cole (1925-26), from the standpoint of the rôle taken by direct action of gravity as leading to tensions produced in muscles which support the organism's weight. With *Helix* Cole was able to show that the speed of upward creeping, after orientation is accomplished, increases with the sine of the angle of inclination of the creeping surface, and thus as the active component of gravity. This leads to the view, substantiated by the effects of forcing such an

animal to carry additional loads (Crozier and Federighi, 1924-25, Cole, 1925-26), that orientation is controlled not by some statocyst function but by the differential gravitational pull upon the two sides of the body (Loeb, 1897),—a view earlier advocated for Chitons (Arey and Crozier, 1919), which lack the statocyst of gasteropods

The information we desired to obtain for the analytical account of geotropism required data upon the amount of upward orientation in a negatively geotropic animal, and the precision of this orientation, as related to the inclination of the creeping surface. For reasons indicated in earlier papers (Crozier and Pincus, 1926-27, *a*, *b*, 1926) we have employed for these experiments young rats of known genetic history, studied during the period of about 2 days which intervenes between the 12th day after birth and the time when the eyelids opened. It happens that with these animals certain new or hitherto ignored features of the geotropic response become apparent and greatly improve the opportunities for investigation. The result seems to indicate quite clearly a direct dependence of orientation upon the distribution of the animal's weight upon the legs of the two sides of the body. Formulæ are derivable describing the orientation with considerable exactness.

We regard it as an interesting fact that, for the first time, a detailed account of a tropism is possible which is based upon experiments with a mammal. This amounts to a sort of reversal of anthropomorphism, and constitutes a decided obstacle for those who would emphasize the greater "simplicity" of lower animals. The simplicity of conduct which permits the mathematical formulation of a mode of behavior is not so much a matter of zoological affinity as it is of dynamical symmetry in the organism and of the choice of experimental conditions which permit the animal to display its potentialities as a machine.

II

In order to record trails of geotropic orientation each rat was placed upon a fine-meshed wire grid, which permitted a good foothold for creeping. When placed on the creeping plane the axis of the body was at first horizontal, or, occasionally, with the head pointing downward. The tilt of the wire surface was measured on a protractor

The angle θ was measured when the animal had oriented and was creeping steadily. The correspondence of the wire grid to coordinate paper made it possible to copy the path upon record sheets. The path was indicated either by marking with chalk the position of the rat's axis on the wire, or by placing a straight-edge parallel to the axis. The central stripe due to the hooding factor facilitated such procedure. The path of orientation is a straight line, as shown diagrammatically in Fig 1, unless, after "hesitation," the rat veers to the opposite side—in which case the angle θ is found to be the same.

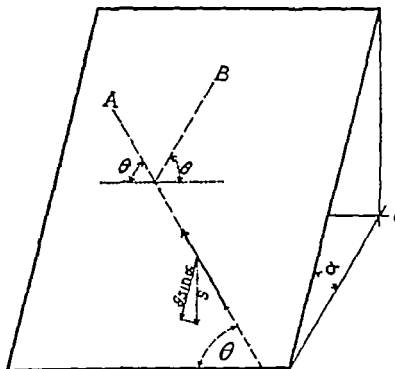


FIG 1 Diagram showing terms used in description of orientation of rats creeping upon a wire grid inclined at α to the horizontal. The position of orientation is defined by the angle θ , the active component of gravity being $G \sin \alpha$. As described in the text, the Path A may be steadily pursued, or the animal may swing to one (B) equally inclined but in the opposite direction.

To obtain data which might be legitimately averaged it is necessary to employ rats as closely comparable as possible. Two albino rats, aged 13 to 14 days, were used in obtaining the records in Fig 7. Those employed for the other measurements (Fig 2, etc.) were of different stock, but litter mates. They were brothers of the seventh backcross generation of King inbred albinos with a dark-eyed stock, and were therefore practically homozygous.

III

The behavior of a rat creeping upon an inclined plane shows one striking peculiarity. It is well known that during the upward locomotion of at least certain negatively geotropic animals the path of progression, especially at inclinations less than 90° , may not be exactly normal to the intersection of the creeping plane with the horizontal. As the inclination is made less, the deviation from the normal increases. This is very obvious in the rats. But there is to be added the further and very important fact that when a rat, at first placed head downward, or with body axis horizontal, orients upward it does so until a certain quite definite angle has been reached, *and then progresses in a straight line*. If creeping becomes interrupted, the rat may show "nervous" random movements of the head. In case these are directed downward, the rat continues creeping along the previous oriented path. But should they be directed upward, locomotion may be pursued at an angle which is exactly the converse of that at first followed. Thus if the angle of orientation was at first 72° to the left, brief creeping may be seen which is more or less irregular but which becomes definite again either at $72^\circ \pm$ to the left, or at $72^\circ \pm$ to the right (*cf* Fig. 1). This clearly points to the limitation of geotropic orientation by a certain threshold determined through the distribution of the gravitational effect upon the two sides of the body. We shall have occasion to return to this point subsequently.

The results summarized in Table I are derived from twenty tests at each inclination, upon each of two rats from the same litter. Individual quantitative differences undoubtedly exist between diverse genetic strains, but since we are not concerned at the moment with this aspect of the matter we have restricted our account to illustrative material free from this source of confusion. The interpretation of data upon other individuals is entirely consistent with that here detailed. The entries in Table I concern (1) the angle of inclination (α) of the creeping plane to the horizontal, (2) the mean angle of orientation in the creeping plane (θ), and (3) the measure of the variability of θ , employing for this purpose the probable error ($0.8534 \Sigma v/n\sqrt{n-1}$) expressed as a percentage of the mean.

It is apparent from Table I that the degree of upward orientation

(θ) increases steadily as the inclination of the creeping plane (α) is made greater, and also that the degree of scatter of the individual readings proportionately decreases—that is, the precision of the orientation is enhanced. The minimum inclination leading to a measurable effect lies between $\alpha = 10^\circ$ and $\alpha = 15^\circ$. At 15° the variability of the measurements of θ is disproportionately high, due presumably to the fact that the threshold effect is intrinsically variable from moment to moment. At values of $\alpha > 70^\circ$, orientation is precisely upward ($\theta = 90^\circ$).

TABLE I

The mean angles of upward orientation (θ) of young rats during creeping upon a surface inclined at angles (α) with the horizontal, and the precision of the respective mean values of θ . The precision is expressed by the probable error as a percentage of the mean (which is equivalent to the coefficient of variation)

α°	θ	Variability of θ per cent
15	32.6°	8.18
20	44.5°	2.27
25	52.9°	1.87
30	57.4°	1.70
35	64.0°	1.41
40	69.8°	1.18
50	77.9°	1.04
60	84.7°	0.529
70	88.3°	0.351

The extent of orientation (θ) is not directly proportional to the gravitational component in the creeping plane, but to its logarithm. The graph in Fig. 2 shows that the equation

$$\theta = K \log (\sin \alpha) \quad (1)$$

gives a satisfactory account of the observations, the goodness of fit is probably due to the fact that the individuals used were very closely comparable.

The extent of orientation as a function of α has been measured in certain molluscs by Davenport and Perkins (1897-98) and by Kanda (1916). In the former paper figures are given for the amount of orientation (θ) corrected for random movement, which is visible in *Limax maximus* after 45 seconds exposure upon an

inclined plane. Essentially this method was also followed by Kanda (1916), who tabulated the percentage of *Littorina* individuals oriented upward after 1 minute exposure. At best, that is with full correction for movements not directed by geotropism, this procedure can give no quantitative expression for the geotropic excitation, for we should need to have, rather, measurements of the times required to produce a given amount of orientation, expressed either as a constant angle (θ) or as a certain percentage of individuals. For this reason little can be gotten from these data. But it is perhaps significant that the amount of orientation, when expressed in this way, increases more rapidly than $\log \sin \alpha$ (Fig. 3).

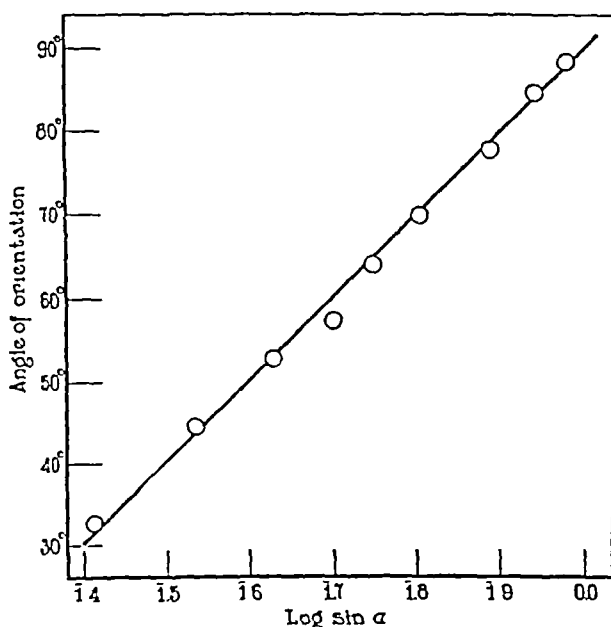


FIG. 2 The angle of upward orientation (θ) is directly proportional to $\log \sin \alpha$, where α is the inclination of the creeping surface. With the exception of one point, the mean values of θ do not depart from the line drawn by more than their probable errors. (The points are averages of 40 measurements.)

This could be understood if the speed of orientation, so measured, should depend upon two things, namely speed of creeping and speed of turning, and if each of these separate elements of the act of orientation (Arey and Crozier, 1921, Crozier and Cole, 1923) should be proportional to $\log \sin \alpha$. For the data of Davenport and Perkins this is very nearly true for values of α above 15° , and for Kanda's figures below $\alpha = 67^\circ \pm$, but not very much weight can be given to the result.

The speed of upward creeping is frequently governed by the intensity of geotropic excitation, and in certain instances can be measured as an index of the effect

of gravity. Cole (1925-26) has done this with *Helix* and concluded that the speed of movement, after orientation is attained, varies as $\sin \alpha$. For the rat as we shall show presently, the velocity of upward movement decreases as $\log \sin \alpha$ increases. Question arises as to the existence of any real difference between the two cases. We believe that there is no real difference, because Cole's data show considerable deviation from $(K)(\sin \alpha)$ at low values of α , and especially for the reason that the speed measured was that of the vertical ascension. The significance of the latter point lies in the fact that the extent of the average orientation (θ , in the

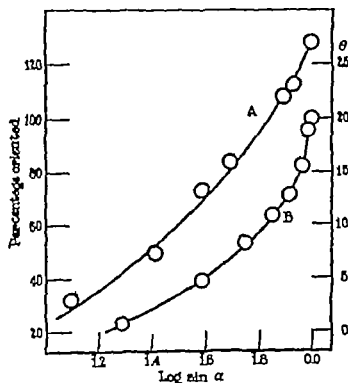


FIG. 3. Curve A. Angle of upward gravitational orientation (θ corrected), for L_{\max} , after 45 seconds (data from Davenport and Perkins, 1897-98). Ordinate scale at the left.

Curve B. One series of measurements ("C") of percentages of oriented individuals after 1 minute (*Lallorina*) ordinate scale at the right. (Data from Kanda, 1916.)

terms previously given) increases with α . Therefore the apparent speed of movement, measured as described, would probably be increased to an illegitimate extent. The data as given show that for *Helix* the "speed of vertical travel" increases faster than $\log \sin \alpha$ (Fig. 4).

We have somewhat regretted the form of the relation $\theta = K \log \sin \alpha$, although there are numerous instances of its applicability to other types of response (*cf.* Hecht, 1919-20, and many further cases), partly

because of its distressing generality, and partly because of its common association with the Weber-Fechner law. The latter interpretation is frequently misleading (*cf.*, especially, Hecht, 1923-24, 1924-25, and, for the case of phototropism under balanced illumination, Crozier,

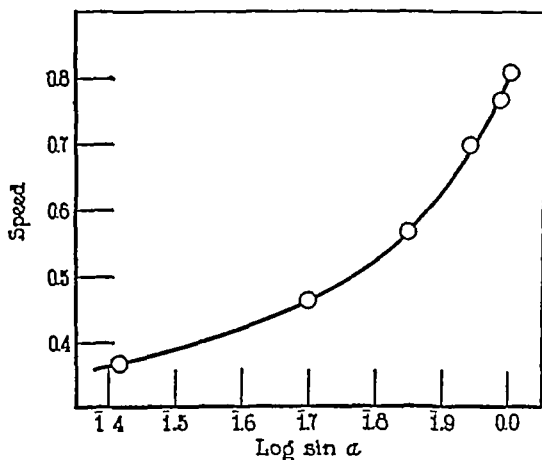


FIG 4 The speed of ascension of *Helix* as related to the inclination (α) of the creeping surface (Data from Cole, 1925-26)

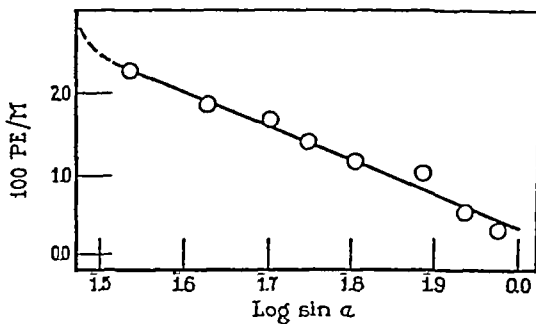


FIG 5 The decrease in the coefficient of variability (P.E. as per cent of the mean), of the measured values of θ (= angle of upward orientation) is proportional to $\log \sin \alpha$

1926-27) But until more is known of this particular phenomenon we may accept the formula as a convenient empirical expression

There is additional evidence of its applicability. If our conception of the orientation is correct, then as the gravitational effect is made greater the *precision* of the upwardly directed movement should be

come correspondingly enhanced. This may be investigated by comparing the numerical expressions for variability of θ as measured at each value of α . If the reduction of variability (V) is proportional to the gravitational stimulus, then

$$-V = K \log \sin \alpha \quad (2)$$

Fig. 5 shows that this relationship is well satisfied, with the exception of the relatively very large variability at $\alpha = 15^\circ$, the deviation here is certainly due to the fact that this inclination is very close to the threshold value for any geotropic effect, as already stated. Thus not only the amplitude or extent, but also the precision of the orientation is determined by the logarithm of the component of gravity acting in the plane of creeping.

IV

In searching for some clue as to the origin of the logarithmic relation between gravitational stimulus and geotropic response we have noted that if attention be paid to the process of creeping during orientation still another relationship emerges. Until a constant value of θ is attained upon a sloping surface the rat is chiefly *pulled* upward by the forward leg of one side of the body and *pushed* upward by the leg of the opposite side, which is less extended. When θ becomes constant the turning moment vanishes. We may consider, roughly and very crudely, that the orienting power is derived from the actions of levers on the opposite sides, and that the lever arm (x) on the "down" side is shorter than that (y) on the "up" side. Then the torque is responsible for turning upward. When the critical angle of orientation is exceeded, the locomotor action on the two sides of the body becomes equalized, so that if this value of θ is definitely exceeded (i.e., beyond a fluctuating zone, of increasing smallness as α is made larger), the rat is no more constrained than upon a horizontal surface, and is free to turn, should it chance to do so, until an equivalent θ is reached on the other side of the perpendicular. We have already described precisely this behavior. In the line of progression defined by θ , the locomotor effectiveness of the opposed limbs is just barely identical. This means that if we assume the axes of the legs to have mean positions perpendicular to that of the body, then $(x \cos \theta - y \cos \theta)$ exactly

balances the total downward pull of the animal's weight, where x and y are the "lengths" of the legs as levers on the two sides. Hence,

$$(x - y) \cos \theta = G \sin \alpha,$$

and

$$\frac{\cos \theta}{\sin \alpha} = \frac{G}{(x - y)}.$$

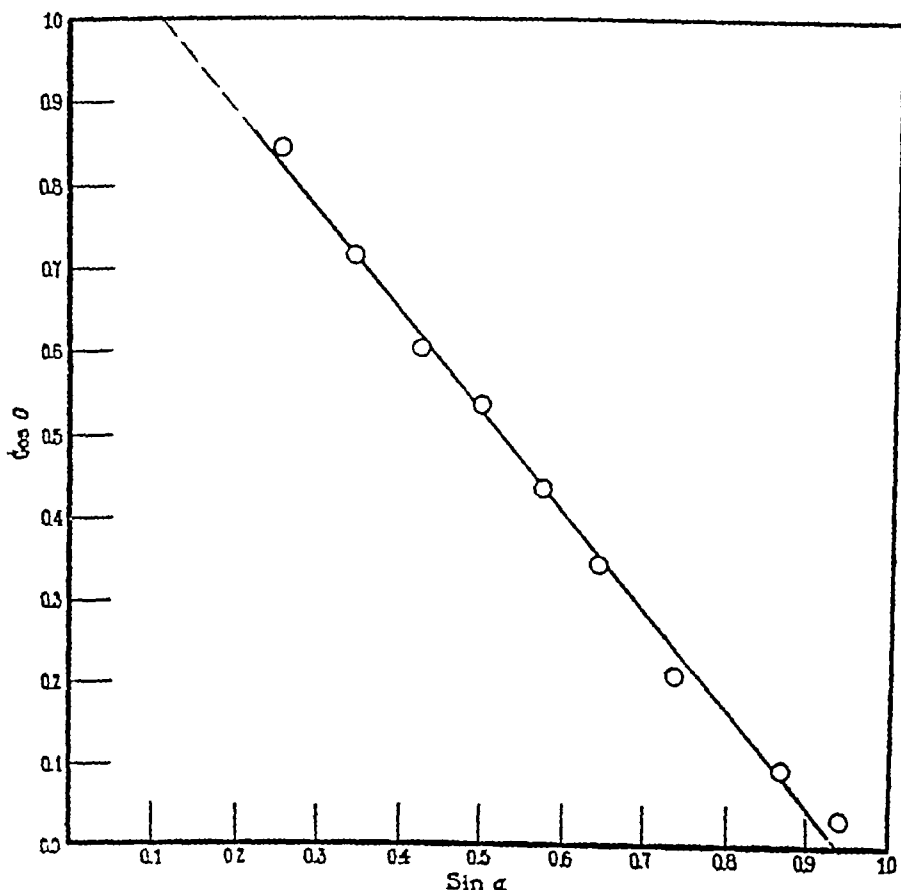


FIG. 6 The cosine of the angle of upward orientation (θ) decreases in direct proportion to the sine of the angle of inclination (α) of the creeping plane

It was pointed out previously that the legs on the upward side are of course more extended, hence, $y > x$, and if $(x - y)$ is constant for all magnitudes of θ , we have

$$-\cos \theta = K \sin \alpha \quad (3)$$

This relationship is in fact displayed, with unexpected exactness, as shown in Fig 6, and gives an independent means of checking the significance of the data summarized in Table I. The constancy of the quantity labelled $(x - y)$, derived from the applicability of (3), at once suggests that the difference between the work done by the limbs of the two sides of the body in lifting the animal's weight must be reduced to constant fraction of the total before a stable orientation is attainable. This does not explain, of course, why the animal orients *upward* rather than downward, which may be determined by the inner ears, but it does explain why the amplitude of orientation attains its particular values as the inclination of the surface is varied, and in our opinion it gives an excellent illustration of the muscle-tension theory of orientation.

V

From Fig 6, by extrapolation to $\cos \theta = 1$, it is found that the ideal threshold value of α is at about 6.5° , at this point the component of gravity in the plane so slightly tilted is $0.113 G$. Experimentally, so far as can be determined, the threshold angle is higher than this (10 – 15°). Hence we may assume, very roughly, because the extrapolation is probably invalid, that when the ratio of the loads on the two sides of the body falls below $10/9$, no further orientation occurs. From the derivation of equation (3),

$$(x - y) \cos \theta = W G \sin \alpha$$

$$\frac{\cos \theta}{\sin \alpha} = \frac{W G}{(x - y)},$$

it follows that if the weight, $W G$, be increased by attaching an additional load with thread to the animal's tail, then, at a given value of α and of θ , the product $(x - y) \cos \theta$ must be larger. The effect of adding such loads is to increase the magnitude of θ , hence it would be expected, from the formula, that $(x - y)$ must increase. The fact is that the locomotion is more labored with added weights attached, and the limbs, especially on the upward side, do become more extended—hence there is good evidence for the occurrence of a change corresponding to an increase in the value of $(x - y)$. The velocity of creeping is decreased in proportion to the added load.

It is of greater interest to see the effect of additional weights upon the extent of the upward orientation (θ). If the effect were a purely mechanical one we would expect to find θ increased in direct proportion to the added weight. But from equation (1),

$$\theta = K \log \sin \alpha,$$

we should expect the change to be such that

$$\theta = K (\log \sin \alpha + \log W \sin \alpha), \quad (4)$$

where W is the added mass, and, when α is constant, θ should increase as $\log W$. Fig. 7 shows that it does. The addition of as little as 1.0

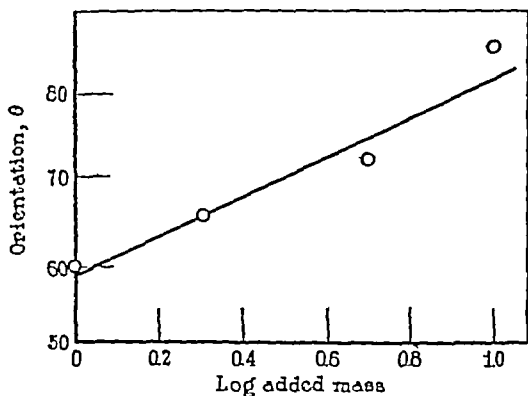


FIG. 7. The angle of upward orientation is proportional to the logarithm of the mass added to the tail of the rat, when the inclination of the creeping plane is constant.

gm increases the upward orientation somewhat. It is worth while to investigate more fully the relations between θ , α , added load, and rate of locomotion, and in a later paper it is proposed to do so.

VI

SUMMARY

Young rats, old enough to creep well but before the eyelids are open, orient and move upward upon an inclined surface. The angle of geotropic orientation on such a surface (θ) is proportional to the logarithm of the component of gravity parallel to the inclined plane.

This result is compared with the scanty information available for other animals, there is indication that it may be generally valid. The precision of the orientation, measured by the percentage dispersion of the individual measurements, also increases in proportion to the logarithm of this component. The cosine of the angle of orientation decreases very nearly in proportion to the sine of the angle of inclination. A possible interpretation of this is given as involving the idea that upward orientation ceases when the differential pull of the body weight upon the opposed legs reaches a threshold value. Attaching weights (W) to the tail causes θ to increase, and in proportion to $\log W$.

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THE PENETRATION OF BASIC DYE INTO NITELLA AND VALONIA IN THE PRESENCE OF CERTAIN ACIDS, BUFFER MIXTURES, AND SALTS

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I.

INTRODUCTION

It has been shown that the pH value of the cell sap plays¹ an important rôle in the accumulation of the basic dye, brilliant cresyl blue, in the living cell of *Nitella*, and in view of this it is important to study the changes in the rate of penetration produced by varying the pH value of the sap. Experiments of this sort, made by McCutcheon and Lucke,² and by the writer,³ showed that the penetration of ammonia increases the pH value of the sap and decreases the rate of penetration of the dye.

The present paper deals with experiments on the rate of penetration of the dye in presence of acids and buffer mixtures. These experiments are of interest in connection with the hypothesis⁴ that brilliant cresyl blue exists in aqueous solution in two forms, called for convenience DB and DS. DB, the form which predominates at higher pH values, represents a free base while DS exists predominantly at lower pH values and is a dissociated salt. A normal living cell of *Nitella* is assumed to be chiefly permeable to DB and only very slightly permeable to DS. The present problem is to find the nature of the factors controlling the penetration of DB.

¹ Irwin, M., *J. Gen. Physiol.*, 1925-26, ix, 561

² McCutcheon, M. and Lucke, B., *J. Gen. Physiol.*, 1923-24 vi 501

³ Irwin, M., *J. Gen. Physiol.*, 1925-26 viii, 147

⁴ Irwin, M., *J. Gen. Physiol.*, 1926-27 x, 75

II

Methods

Only general methods will be given here special methods for each set of experiments will be described in connection with the results

The experiments were carried out in an incubator at $25^{\circ}\text{C} \pm 0.5^{\circ}$ having air holes through which diffused light entered

Only living cells were used In order to obtain cells in the same condition for experimentation uniformity as to length, thickness, and external appearance was attended to In the case of *Nitella flexilis* the cells used were taken from the central portion of the plant, midway between the tip and the root Seasonal changes bring about differences in the permeability of the cells, so that a series of comparative experiments were made on the same lot of cells collected within a short period (near New York in spring unless otherwise stated)

A control experiment was always carried out by removing cells directly from tap water and placing them in the same dye solution as in the case of the test experiment (in which the cells were given some special treatment before being placed in the dye) The rate of penetration obtained from the control experiment was used as a standard of comparison in order to determine the change in the rate of penetration of the dye caused by varying the media in which the cells were placed previous to exposure to the dye solution

Every determination given represents an average of over 60 experiments and the probable error of the mean is in all cases less than 7 per cent of the mean

In the case of *Valonia macrophysa* (collected in Bermuda), the procedure was as follows The clusters of cells were pulled apart and the individual cells were allowed to stand in pans of sea water (which was changed daily) for over 2 weeks in the laboratory (exposed during the day to diffused light) During this period the cell wall at the point of detachment thickened somewhat This precaution was taken to diminish irregularity in the rate of penetration and the susceptibility of cells to injury upon exposure to solutions

Small cells (each having a volume of about 0.1 cc) with one point of detachment, and having practically no attached cells, were chosen Care was taken to remove adhering organisms or deposits from the surface of the cell

The detection of an early stage of reversible injury is a very difficult matter, especially with *Valonia* In the case of *Nitella* an increase in the rate of accumulation of the dye may serve as an indication of a preliminary stage of an injury under certain conditions but this does not seem to be markedly evident in *Valonia* The exit of halides from the vacuole of *Nitella* or the entrance^{5,6} of SO_4 into *Valonia* seems to indicate advanced stages of injury

⁵ Osterhout, W. J. V., *J. Gen. Physiol.*, 1925-26, viii, 131

Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, ix, 255

⁶ Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360

Cells in good condition are turgid as they become injured the turgidity diminishes. An experienced experimenter can, to a certain extent, predict the degree of resistance of the cells of *Nitella* and *Valonia* to experimental treatment by the turgidity as ascertained by touching them. This method was used by the writer as a rough guide to the condition of the cells but it does not serve to tell whether injury is reversible. The criterion of irreversibility of injury employed by the writer was as follows: cells which had been exposed to experimental conditions were replaced in the normal medium (*Nitella* in tap water *Valonia* in sea water) and at intervals during 2 days the rate of mortality was compared with that of the control cells (without exposure to experimental conditions). The criterion of death for *Nitella* was a complete and permanent loss of turgidity, and for *Valonia* either a complete collapse of the cell or disarrangement of chlorophyll and its appearance in the vacuole, so that the greater part of the cell surface appeared colorless. Another test of the condition of the cells is to observe the length of time it takes for them to die in the experimental solutions.

It is not possible, however, to determine experimentally whether the cell was injured at the time of experiment unless the injury happened to be irreversible. By using these tests an attempt was made to keep the cells uniform during the experiments.

The dye used was made by Grüber and was dissolved in buffer solutions (1/150) in the case of *Nitella* and in sea water in the case of *Valonia*. The pH values of the solutions determined colorimetrically were checked as much as possible by means of the hydrogen electrode. Solutions were not stirred unless otherwise stated.

The determination of the concentration of the dye in the sap was made colorimetrically. With *Nitella* the cell was gently wiped and was cut at one end, so that the sap could be squeezed out onto a glass slide. With *Valonia* the surface of the cell was punctured with a sharp capillary tube and the sap was drawn up from the vacuole into the tube from which it was pushed out onto a glass slide. In both cases the sap was drawn up into capillary tubes and the color was matched with capillary tubes of the same diameter containing standard dye solutions.

To determine the pH value of the sap a definite volume was taken by filling a tube for 2 inches with the sap. Indicator solution was drawn up into another tube for a distance of $\frac{1}{10}$ of an inch. The contents of both these tubes were pushed out onto a glass slide and thoroughly mixed. This mixture was then drawn up into a capillary tube and the color matched with that of the capillary tube containing a mixture of standard buffer solution at a known pH value and the same amount of the indicator (the mixture was prepared in the same manner as in the case of the sap). Care was taken to have the least possible contamination of the sap by CO_2 from the breath of the experimenter as well as to prevent escape of CO_2 into the air as far as possible.

The color of the indicators changed on standing in a buffer solution containing artificial *Valonia* sap, and also on standing in the natural expressed sap but the color of the indicators did not change during the time required to determine the pH value of the sap.

The salts in the sap of *Nitella* (about 0.1 M halides) do not seem to affect the indicator seriously, but those in the sap of *Valonia* (about 0.6 M halides) have a very definite effect. In view of the fact that we know so very little about the salt error in general, and possible specific effects of individual salts on these indicators, it will be necessary to study this question carefully before absolute pH values of the sap of *Valonia* can be given.

Another possible source of error in the case of *Valonia* is that the sap is so little buffered that an addition of indicator solution may bring about a change in the pH value of the sap, careful experiments must therefore be made to avoid this error. On the other hand, the sap of the *Nitella* used by the writer is buffered so that this source of error may be negligible. Since only approximate and relative values are desired the pH values of the sap of *Valonia* and *Nitella* given in this paper represent values without a correction for salt error, determined by means of one concentration of indicator dissolved in distilled water of pH 5.8 (approximately the pH value of the sap), or indicator dissolved in alcohol (methyl red). For one series of changes in the pH values only one indicator is used. For example, when experiments were made by exposing cells to a solution of NH_4Cl , brom-cresol purple was used; in the case of cells placed in acid solutions methyl red was used. Brom-cresol green was used to check the values obtained with methyl red, but in view of the fact that the color above pH 5.2 was not satisfactorily matched, only a very rough estimation of the pH value of the normal sap could be made by this indicator. Each indicator is taken from the same stock solution for each series of experiments.

III

The Decrease in the Rate of Penetration of Dye When the pH Value of the Sap Is Lowered by Entrance of Acetic Acid

The cells were divided into four lots. One lot was placed in an acetate buffer mixture at pH 5.1, and at the end of 10 minutes the pH value of the sap was compared colorimetrically with that of the normal cell sap. It was found to have decreased⁷ from pH 5.5 (normal) to pH 4.9.

⁷ It may be added here that these experiments show that acetic acid enters the vacuole rather easily from an acetate buffer mixture and decreases the pH value of the sap until the internal pH value is less than the external. This agrees with the results obtained by many investigators showing that weak acids enter the living cells. The writer's experiments also show that the pH value of the sap may be raised again when acetic acid is allowed to come out of the vacuole by placing the cells in a solution containing no acetic acid (the more alkaline the external pH value, the more rapid is the rate of exit of acetic acid from the vacuole).

The second lot of cells was placed in the acetate buffer mixture at pH 5.1, and after 10 minutes they were removed, wiped, rinsed for 5 seconds in phosphate buffer mixture at pH 6.6, wiped, and placed in fresh phosphate buffer mixture at pH 6.6. After 1 minute the cells were removed, and the pH of the sap was determined. It was found to be pH 5.2, which is 0.3 pH lower than that of the normal cell sap.

TABLE I.

Comparison of the amount of brilliant cresyl blue in the vacuole when the living cells of *Nitella* are placed for 1 minute in 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) after previous exposure to M/150 acetate buffer solution at pH 5.1 for different lengths of time. The rate of penetration of dye in the case of cells directly transferred from the tap water to the dye solution is used as the standard of comparison.

External solutions.		In tap water at pH 7.7	In acetate buffer solution 5 sec.	In acetate buffer solution 1 min.	In acetate buffer solution 10 min.
When dye solution is not stirred or changed.	Amount of dye in sap	M 0.000073	M 0.000069	M 0.000069	M 0.000037
	Percentage decrease on basis of 0.000073 as standard.		5 per cent	5 per cent	50 per cent
When dye solution is stirred and changed every 5 sec.	Amount of dye in sap	M 0.00012			M 0.000056
	Percentage decrease on basis of 0.00012 as standard				47 per cent

The third lot of cells was first exposed to the acetate buffer solution at pH 5.1 for 10 minutes, after which they were removed, wiped, washed for 5 seconds in phosphate buffer mixture at pH 6.6, again wiped, and placed in the 0.00035 M dye solution at pH 6.6 (phosphate buffer mixture). After 1 minute they were removed from the dye solution and the concentration of the dye in the sap was determined colorimetrically, and was found to be 0.000037 M.

The fourth lot of cells was taken directly from the tap water (at pH 7.7) and placed in the same dye solution as the third lot of cells. At the end of 1 minute the concentration of the dye in the sap was found to be 0.000073 M.

Cells thus treated did not live so well as the control cells when replaced in tap water so that in all probability they were more or less injured, but during the experiment the actual appearance of the cells, in respect to chlorophyll arrangement and turgidity, seemed about the same as that of control cells except that the sap appeared slightly murky. Cells kept continuously in the acetate buffer solution began to die in about 3 hours, so that after an exposure of 10 minutes there may have been a very slight injury.

Thus these experiments show that the decrease in the pH value of the sap brought about by acetic acid may be associated with a decrease^{8,9} in the rate of penetration of dye amounting to about 50 per cent, as shown in Table I.

This fact is of particular interest in connection with the theory^{1,4} that the dye is chiefly in the form of free base (for convenience called DB), at high pH values, and that this alone can penetrate the proto-

⁸ This decrease in the rate of penetration of dye is not due to the lowering of pH value of the external solution immediately surrounding the cell wall as a result of diffusion of acetic acid from the vacuole, because when the experiment is repeated by stirring the external solution, the relative amount of decrease in the rate is about the same as when the external solution is not stirred, as shown in Table I. Furthermore, this decrease is not caused by the adhering of acetic acid to the surface of cell wall in such a manner that it cannot be removed by washing and wiping before the cells are placed in the dye solution, because when the cells are placed in the dye solution, after they have been dipped in the acetate buffer solution only for 5 seconds or for 1 minute instead of 10 minutes, during which exposure the pH value of the sap remains normal, there is no decrease in the rate of penetration of dye, as shown in Table I.

⁹ This result is contrary to the result obtained with Cambridge *Nitella* previously described (Irwin, M., *J. Gen. Physiol.*, 1925-26, ix, 566, Foot-note 11) where an increase in the rate of penetration took place, but the extent of this increase was not so great (about 25 per cent). Since this work on Cambridge *Nitella* was done in midwinter, the experiments were repeated with the cells obtained in the summer, and it was found that in the majority of cases a decrease took place (about 25 per cent), which is less than the decrease in the case of New York *Nitella*. Such a difference in the behavior of cells may be due to the difference in the condition of the protoplasm.

plasm and enter the vacuole, and that the extent of accumulation of the total dye is dependent on the extent of change of this form, DB, on entering the vacuole into another form, DS, which cannot pass through the protoplasm. In that case we might expect the rate of penetration into the vacuole to be increased when the pH value of the sap is decreased, since with this decrease in the pH value of the sap the ratio of DB/DS decreases in the sap so that as DB enters the vacuole more of it will change to DS, thus causing more DB to enter. But since the experimental results give evidence to the contrary it is evident that the factor which controls the rate of penetration of dye into the vacuole cannot be wholly dependent on the condition of the sap. Under the present experimental conditions the rate of penetration of the dye must be controlled primarily by the effect of the acetate buffer on some other part of the cell. A series of experiments was therefore undertaken to determine the cause of this decrease in the rate of penetration of dye into the vacuole.

IV

Can the Decrease in the Rate of Penetration of the Dye be Produced without Change in the pH Value of the Sap?

If the theory⁴ outlined in Sections I and III were correct we might assume that the decrease in the rate of penetration of dye associated with a decrease in the pH value is due to a change either at the surface or inside the protoplasm caused by the acetate buffer mixture. In that case we might very well expect a decrease in the rate of penetration when the cells are exposed¹⁰ to the solution only long enough for the protoplasmic surface or the interior of the protoplasm to be affected before a change in the pH value of the sap occurs. Unfortunately it is not possible to use the acetate buffer solution for this purpose since the pH value of the sap changes after a very few

¹⁰ The detailed description of the method of experimentation will be omitted hereafter since it is given in Section III. It may be repeated here that in all cases the cells were washed for about 5 seconds in a buffer solution at the same pH value as that of the dye solution before they were placed in the dye solution and the cells were invariably wiped before they were placed in any solution. Cells were exposed for 1 minute in the solution of dye 0.00035 M made up with phosphate buffer mixture at pH 6.6 unless otherwise stated.

Amount of dye in sap	μ 0.000073	μ 0.000073	μ 0.000071
Percentage decrease on basis of 0.000073 μ standard		47 per cent	44 per cent

amount of decrease¹¹ in the rate of penetration whether the pH value of the sap is lowered or remains normal. The mortality of the cells thus treated is lower than that of the cells exposed to the acetate buffer mixture.

¹¹ Since there is about the same amount of decrease in the rate of penetration of dye whether the pH value of the sap is decreased or not, such a decrease cannot be due primarily to the decreasing of the pH value of the film⁴ of liquid between the protoplasmic surface and the cell wall as result of diffusion of acetic acid from the vacuole into the film. This film is the only part of the external system which affects penetration since it alone determines the number of dye molecules striking the protoplasmic surface. The condition of the external solution may be regarded as of importance only in so far as it affects this film.

V

Is the Decrease in the Rate of Penetration of the Dye Due to the Effect of H Ions on the Surface or to Their Penetration (as Ions) or to the Entrance of Acids in Undissociated Form?

The decrease in the rate just described was about the same whether the pH value of the sap was lowered or not, and this suggests that the decrease in the rate might be due to the direct action of H ions on the surface or their penetration as ions when the pH value of the external solution changed from pH 7.7 (tap water) to pH 5.4 (buffer solutions). If this assumption were correct we might expect the rate of penetration to be about the same whether the cells were previously exposed to tap water, to phosphate, or to borate buffer solutions at pH 7.7 providing equal numbers of hydrogen or hydroxyl ions enter in each case.

In order to test this the rates of penetration of dye were compared among the three groups of cells previously placed¹⁰ for 10 minutes (1) in tap water (control), (2) in phosphate buffer solution, and (3) in borate buffer solution, all at pH 7.7, and it was found (as shown in Table III) that with phosphate buffer solution there was about 30 per cent less dye in the vacuole than in the case of the control, and with borate buffer about 13 per cent less dye (which may not be significant since the probable error of the mean is rather high).

The experiments were extended to higher pH values, pH 8.1 and 7.3, and it was found, as shown in Table III, that the rate of penetration of dye is again lower in the case of cells previously exposed¹⁰ to the phosphate buffer solution than that in the case of cells exposed to the borate solution. Such a difference in behavior between the borate and the phosphate buffer mixtures cannot be due to the effect of H or OH ions as such on the cell, since the pH value is the same in both these solutions.

Other experiments are therefore needed to determine just what causes this difference.

This difference between the phosphates and the borates, as affecting the rate of penetration of dye, is not due to the difference in the effect¹²

¹² It is not possible, unfortunately to determine if there is an effect of acetate buffer mixture on the dye, since it is impossible to determine the penetration of dye at a pH value lower than pH 6.2 in the case of *Nitella*.

of these buffer mixtures directly on the dye, as is proved by the fact that when the cells are transferred¹⁰ directly from the tap water to

TABLE III

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella*, when cells were previously exposed to M/150 borate and phosphate buffer solutions at different pH values for 10 minutes after which they were placed for 1 minute in 0.00035 M dye solution at pH 6.6 (1/150 phosphate buffer mixture)

External solutions.	In tap water at pH 7.7	In phosphate buffer solution at pH 7.7	In borate buffer solution at pH 7.7	In phosphate buffer solution at pH 8.1	In borate buffer solution at pH 8.1
	M	M	M	M	M
Amount of dye in sap	0.000079	0.000055	0.000069	0.000059	0.000079
Percentage decrease on basis of 0.000079 as standard		30 per cent	13 per cent	26 per cent	0 per cent

External solutions.	In phosphate buffer solution at pH 7.3	In borate buffer solution at pH 7.3	In phosphate buffer solution at pH 6.6	In borate buffer solution at pH 8.7	
	M	M	M	M	
Amount of dye in sap	0.000052	0.000069	0.000048	0.000079	
Percentage decrease on basis of 0.000079 as standard	35 per cent	13 per cent	39 per cent	0 per cent	

TABLE IV

Comparison of the amount of brilliant cresyl blue in the sap after 1 minute in 0.00017 M dye solutions at pH 7.7 made up with different buffer mixtures (M/150)

External dye solutions.	Borate buffer mixture	Phosphate (ordinary) $\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$	Phosphate (lacking K) $\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$
	M	M	M
Amount of dye in sap	0.00041	0.00035	0.00040

the dye solution at pH 7.7 made up (1) with borate buffer mixture and (2) with phosphate buffer mixture, the rate of penetration of the dye is the same, as shown in Table IV

This difference furthermore is not due to a specific action of the K in the phosphate buffer mixture since the experiments were repeated¹⁹ with the solution made up with Na_2HPO_4 containing NaH_2PO_4 instead of KH_2PO_4 , at pH 5.4 and 7.7 and the same result was obtained, as shown in Table V

TABLE V

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella* after 1 minute exposure to 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) following a 10 minute exposure to the M/150 phosphate buffer mixtures consisting of Na_2HPO_4 and either KH_2PO_4 or NaH_2PO_4

External solutions.	In tap water at pH 7.7	In Na_2HPO_4 + KH_2PO_4 at pH 7.7	In Na_2HPO_4 + NaH_2PO_4 at pH 7.7	In Na_2HPO_4 + KH_2PO_4 at pH 5.4	In Na_2HPO_4 + NaH_2PO_4 at pH 5.4
Amount of dye in asp.	M 0.000084	M 0.000055	M 0.000059	M 0.000048	M 0.000048
Percentage decrease with 0.000084 as standard		33 per cent	29 per cent	42 per cent	42 per cent

TABLE VI.

Comparison of amount of brilliant cresyl blue in the vacuole when cells of *Nitella* (autumn) are placed in 0.00004 M dye solution (stirred) at pH 7.7 (M/150 borate buffer) for $\frac{1}{2}$ minute, after they have been exposed for 10 minutes to various solutions.

External solutions.	Tap water pH 7.7	Boric acid pH 4.8.	Hydrochloric acid pH 4.8.	Phosphoric acid pH 4.8.	Phosphate buffer pH 5.4
Dye in asp.	M 0.000072	M 0.000076	M 0.000069	M 0.000058	M 0.000042
Percentage decrease on basis of 0.000072 as standard.		5 per cent increase (?)	5 per cent	20 per cent	42 per cent

The inhibiting effect of phosphate buffer mixtures is greater the lower the pH value, as shown in Table III. It may be that this is due to the greater amount of phosphoric acid present in the buffer mixture, if we assume that as a weak acid it penetrates the protoplasm as undissociated molecules and dissociates after entering and

lowers the pH value of the protoplasm, so that when cells are subsequently placed in a dye solution there will be less DB (since DB changes to DS more at a low pH value) in the protoplasm than in the case of the control cells which are transferred directly from tap water to the dye solution. The rate of penetration of DB from the protoplasm to the vacuole will therefore be less than in the case of the control cells.

This assumption¹² is partly supported by the following result. When the cells¹³ are exposed for 10 minutes to three separate solutions, (1) hydrochloric acid, (2) boric acid, (3) phosphoric acid, all at pH 4.8, and then placed in the dye solution¹³ (borate) for $\frac{1}{2}$ minute, the rate of penetration of dye (as compared with that of the control cells, which are transferred directly from tap water to the same dye solution) in the case of hydrochloric acid and boric acid is about the same as that of the control. This indicates that H ions do not affect¹² the cell and that if boric acid enters the cell as undissociated molecules it does not afterward dissociate sufficiently to lower the pH value to any appreciable degree. Phosphoric acid behaves differently in that the rate of penetration of the dye in the case of the cells exposed previously to this acid is found to be about 20 per cent lower than that of the control, which indicates that phosphoric acid enters the

¹² There are several other possible explanations, for example

(1) On the basis that phosphoric acid enters more rapidly than boric. We are unable to prove this experimentally, for which reason the explanation described in the text is used instead.

(2) On the basis that a weak acid enters the protoplasm as undissociated molecule and by dissociating lowers the pH value of the protoplasm and that when such cells are removed from the buffer solution to the dye solution, the weak acid diffuses out from the protoplasm into the film of liquid between the protoplasmic surface and the cell wall, and lowers the pH value of the film thereby decreasing the ratio of DB/DS in the film. This will explain the difference between boric acid and phosphoric acid, in that boric acid does not change the pH value of the film since it is too weak an acid, while phosphoric acid is sufficiently strong to bring about this change. But this assumption is not so satisfactory as the one described in the text when we consider the fact that there is an inhibiting effect on the rate of penetration of dye even with cells previously exposed to a phosphate buffer solution at pH 8.1 and then placed in dye solution at much lower pH value (pH 6.6). In such a dye solution one would expect further entrance of phosphoric acid into the cell, rather than exit of the acid from the protoplasm to the exterior of the cell. These cells were collected in autumn.

The dye solution was stirred. At a lower pH value both phosphoric acid and hydrochloric acid have an inhibiting effect which is greater in the case of the former.

protoplasm and then dissociates sufficiently to lower the pH value or else that it has a specific effect on the surface (Table VI)

The cause of the decrease brought about by the phosphate buffer mixture may be threefold, (1) due to undissociated phosphoric acid, (2) due to the Na and K salts present in the buffer mixture, and (3) due possibly to certain anions

It may be of interest to add here the following When cells (collected in Cambridge) are exposed for 10 minutes to solutions at different concentrations (0.05 M to 0.006 M) of NaCl, LiCl, KCl, Na₂SO₄, and NaNO₃ made up in distilled water, after which they are washed in distilled water for 5 seconds, wiped, and are placed in 0.00014 M dye solution at pH 7.7 (borate buffer mixture) for 1 minute, the rate of penetration is considerably decreased as compared with the control. If cells are placed directly for 1 minute (without such treatment) in 0.00014 M dye solution at pH 7.7 (borate buffer mixture) containing any one of these salts, the rate is found to be slightly higher than in the case of cells placed in dye solution containing no salt.

Solutions of MgCl₂, MgSO₄, CaCl₂, LaCl₃, and LaNO₃ all behave alike, in that when cells are exposed to these solutions for 10 minutes and then transferred to the dye solution, the rate of penetration of dye is about the same as the control. When cells are placed without such treatment in dye solutions containing any one of these salts (LaCl₃ omitted), the rate is found to be somewhat higher than that of the control

Thus there is evidence for the inhibiting effect¹⁴ of the salts with

¹⁴The experiments described in the text (see Table III) show that the borate buffer mixtures have no inhibiting effect on the rate of penetration of dye. In view of the fact that the borate buffer mixtures at higher pH values contain a considerable amount of Na, there is an apparent discrepancy between the results obtained in this case and those in the case of NaCl solutions in which there is a considerable inhibiting effect due to the presence of Na (this discrepancy was mentioned in the writer's previous paper (Irwin M *Proc Soc Exp Biol and Med*, 1926, xxiv No. 1)) This however may be due to the fact that in the case of cells previously exposed to the borate buffer mixtures the dye was made up with phosphate buffer mixtures which seem to diminish the inhibiting effect of Na, while in the case of cells previously exposed to NaCl solutions the dye used was made up with borate buffer mixture which does not seem to have this effect. The following experiments may make this clear When cells previously exposed to (1) 0.01 M NaCl and (2) to 0.005 M sodium borate solutions for 10 minutes were washed for 5 seconds in distilled water, wiped, and placed in 0.00014 M dye solution at pH 7.7 (borate buffer mixture), there is a considerable inhibiting effect

monovalent base cations which is not easily reversible, since the effect may be brought about by placing the cells in 0.01 M NaCl solution for 5 minutes, but this effect does not disappear after the cells have been transferred to distilled water and left for over an hour. This effect, however, may be readily removed if cells are placed in a solution of salt with bivalent or trivalent cations, such as $MgCl_2$ and $LaCl_3$ at certain concentrations.

Further experiments are being carried out on this subject by the writer.

VI

Experiments on Valonia

The experiments have been repeated with *Valonia macrophysa* but the results described below are approximate and show only relative values, owing to the fact that the pH value of the sap cannot be accurately determined without special experiments and that the sea water not only shifts the dissociation constant considerably, but seems to change the nature of the dye, especially at lower pH values.

A The Effect of Aqueous Ammonia (Free and Combined)—Since the method of determining the change in the pH value after placing cells in solutions has been described in detail in Section II, it will be omitted here. When cells of *Valonia* were placed for 1 hour in sea water containing 0.003 M NH_4Cl solution, the pH of the sap increased from 6 (normal) to 6.6 (determined colorimetrically by using brom-cresol purple). When such cells were replaced in sea water and left for 1 hour the pH value decreased from 6.6 to 6.1.

One group of cells was placed in sea water containing 0.00035 M dye, a second group in sea water containing 0.003 M NH_4Cl and

which is slightly greater with (1) than with (2). If such cells (1) and (2) are placed in 0.00014 M dye solution at pH 7.7 (phosphate buffer solution) they show no inhibiting effect at all.

At a higher concentration of NaCl (0.05 M) this inhibiting effect is not removed in 0.00014 M dye made up with phosphate buffer mixture at pH 7.7.

The inhibiting effect of previous treatment with the phosphate buffer mixture at pH 5.4 (Table II) is increased in 0.00014 M dye solution at pH 7.7 made up with borate buffer mixture.

0.00035 M dye. A third group of cells was first exposed for 1 hour to sea water containing 0.003 M NH_4Cl and then transferred to the dye solution used in the case of Group 1. After 1 hour there was a decided decrease in the rate of penetration of dye in the case of cells placed in the dye solution containing NH_4Cl (Group 2) and also in the case of cells previously exposed to NH_4Cl solution (Group 3), as compared with the control (Group 1). These results show that the presence of ammonia in the cell brings about a decrease in the rate of penetration of dye. Whether this decrease is entirely due to the increase in the pH value of the sap in the presence of ammonia or due partly to the former and partly to the presence of ammonia in the protoplasm (at the surface or the interior), it is not possible to determine. These results confirm those obtained with *Nitella*² (see Section I).

B. Effect of Acetic Acid and HCl at pH 5.9—Let us first see if the same results may be obtained as with *Nitella* when the pH value of the sap is decreased by entrance of acetic acid. When cells were placed in sea water containing acetic acid at pH 5.9, the pH value of the sap decreased in 1 hour from 5.5 (normal) to 4.8 (methyl¹⁸ red used as an indicator). The pH value of the sap thus decreased was found to be raised to the normal when such cells were placed in sea water for 20 minutes. When cells whose pH value had been thus decreased were placed for 20 minutes in sea water containing 0.00035 M dye, the amount of dye in the sap was less¹⁸ than in the sap of cells transferred directly from the sea water to the same dye solution (control). These experiments show that there is a decrease in the rate of penetration of dye when the pH value of the sap is decreased.

¹⁸ Difference between the determination of the pH value of the sap made with brom-cresol purple and with methyl red lies in the fact that the effect of salt on the indicator is not corrected. The explanation of the use of the indicators is described in Section I.

¹⁹ Brooks exposed cells of *Valonia macrophylla* to sea water (1) containing NH_4Cl until the pH value of the sap increased, and (2) containing CO_2 until the pH value of the sap decreased, after which they were placed in sea water containing 2, 6, dibromophenol indophenol, and found that the rate of penetration of dye decreased with (1) and increased with (2). She interprets these results on the basis that the rate of penetration of dye is affected by the change in the pH value of the external solution surrounding the cell as a result of diffusion of (1) NH_4Cl and (2) CO_2 from the vacuoles. (See Foot note 6.)

by entrance of acetic acid, which agrees with the results discussed in Section III on *Nitella*

Let us now see if a decrease can be brought about without a change in the pH value of the sap. Cells were exposed for 1 hour to sea water containing HCl at pH 5.9, after which they were placed in 0.00035 M dye for 20 minutes. The rate of penetration in this case was found to be less than the control but the extent of decrease in the rate is not so great as it was in the case of cells exposed to acetic acid.

When cells are placed for 1 hour in sea water containing 0.0007 M dye at pH 5.9, (1) containing acetic acid and (2) containing HCl, the rate of penetration was found to be higher with acetic acid than with HCl.

C. Effect of Sea Water at pH 6.5 Containing either Acetic Acid or HCl (No Change in the pH Value of the Sap)—The question now arises as to what will happen if we put cells in sea water containing acetic acid at a pH value at which there is no decrease in the pH value of the sap. Cells were placed in sea water at pH 6.5 containing acetic acid for 1 hour after which they were transferred to sea water containing 0.00035 M dye for 20 minutes. The pH value of the sap remained normal. When the rate of penetration of dye in the case of the cells thus treated was compared with that of the control (cells directly removed from the sea water and placed in the same dye solution), it was found to be the same. In the case of the cells previously exposed to sea water containing HCl at pH 6.5 the rate of penetration of dye was also found to be the same.

Cells placed in 0.00017 M dye in sea water at pH 6.5 containing (1) acetic acid and (2) HCl, showed no difference in the rates.

Thus these experiments show that in the case of *Valonia* also the rate of penetration of dye may be retarded when (1) the pH value of the sap is decreased in presence of acetic acid, and (2) the pH value of the sap is increased in presence of NH_3 , when cells are exposed to these solutions before they are placed in the dye solutions.

SUMMARY

When living cells of *Nitella* are exposed to an acetate buffer solution until the pH value of the sap is decreased and subsequently placed in a solution of brilliant cresyl blue, the rate of penetration of dye into the vacuole is found to decrease in the majority of cases,

and increase in other cases, as compared with the control cells which are transferred to the dye solution directly from tap water. This decrease in the rate is not due to the lowering of the pH value of the solution just outside the cell wall, as a result of diffusion of acetic acid from the cell when cells are removed from the buffer solution and placed in the dye solution, because the relative amount of decrease (as compared with the control) is the same whether the external solution is stirred or not.

Such a decrease in the rate may be brought about without a change in the pH value of the sap if the cells are placed in the dye solution after exposure to a phosphate buffer solution in which the pH value of the sap remains normal. The rate of penetration of dye is then found to decrease. The extent of this decrease is the greater the lower the pH value of the solution.

It is found that hydrochloric acid and boric acid have no effect while phosphoric acid has an inhibiting effect at pH 4.8 on stirring.

Experiments with neutral salt solutions indicate that a direct effect on the cell (decreasing penetration) is due to monovalent base cations, while there is no such effect directly on the dye.

It is assumed that the effect of the phosphate and acetate buffer solutions on the cell, decreasing the rate of penetration, is due (1) to the penetration of these acids into the protoplasm as undissociated molecules, which dissociate upon entrance and lower the pH value of the protoplasm or to their action on the surface of the protoplasm, (2) to the effect of base cations on the protoplasm (either at the surface or in the interior), and (3) possibly to the effect of certain anions. In this case the action of the buffer solution is not due to its hydrogen ions.

In the case of living cells of *Valonia* under the same experimental conditions as *Nitella* it is found that the rate of penetration of dye decreases when the pH value of the sap increases in presence of NH_3 , and also when the pH value of the sap is decreased in the presence of acetic acid. Such a decrease may be brought about even when the cells are previously exposed to sea water containing HCl , in which the pH value of the sap remains normal.

The writer wishes to thank Miss Helen McNamara for her faithful assistance in carrying out the experiments.

THE RÔLE OF CERTAIN METALLIC IONS AS OXIDATION CATALYSTS

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It has long been known that metals in some way are very closely connected with the processes of respiration and oxidation in both plants and animals, and the mode of their action has been the subject of a great deal of research and discussion. The most important of these metals is undoubtedly iron, and the recent work of Warburg (1925) and Meyerhof (1924) has gone far toward elucidating its mode of action¹. Iron as an oxygen carrier is present in the hemoglobin of the blood of mammals and has been the subject of much investigation. It is not within the province of this paper, however, to discuss this aspect of the matter, treatment of which may be found in text-books of general physiology.

Next to iron, in occurrence and in importance is copper although the latter has not received the careful and attentive research which has been bestowed on the former².

¹ The book by Meyerhof (*Chemical dynamics of life phenomena* 1924) contains an excellent summary of investigations up to a very recent date. Reference may also be made to the individual papers of these authors, and particularly, in English of Warburg (1925). Crozier (1924-25) has analyzed the data of various investigators who have worked with oxidation systems from the point of view of the effect of temperature. He finds that many oxidation reactions, including several which are undoubtedly catalyzed by iron, have a critical thermal increment close to 16,000. Hecht (1925-26) explains on this basis the latent period of the photic response in *Ciona*, the critical thermal increment of which is nearly 16,000, as a reaction catalyzed by iron and probably an oxidation. The fact that many oxidation systems and many reactions catalyzed by iron have the same increment may be taken as further evidence that iron is intimately connected with oxidation catalysis.

² The toxic effects of copper have been widely observed and discussed (see papers by Cook, 1925-26). In fact, the striking and obvious toxic action of copper has more or less obscured the possibility that this element may be of fundamental importance in the catalysis of normal oxidation reactions.

That copper occurs normally in a large number of organisms has been shown by Maquenne and Demoussy (1920) for plants and Muttkowski (1920-21) and Rose and Bodansky (1920) for animals. In animals it is usually found in the hemocyanin, or oxygen-carrying fluid, of arthropods and crustaceans. Hemocyanin is considered analogous to hemoglobin, with copper taking the place of the iron. Henze (1904-05), working on octopus blood, decided that oxidations were catalyzed by the copper. Alsberg and Clark (1914), using *Limulus*, concluded that "With the aid of copper, oxygen may, perhaps, be transferred catalytically within the organism." Glaser (1923) found considerable copper in *Arbacia* eggs, he thinks its function is partially to inactivate certain enzymes.

Of the other metallic elements manganese (Bertrand, 1897, and later papers) is the only one which has been mentioned as a substitute for iron and copper. McHargue (1926) states that manganese is found very frequently in chlorophyll-bearing tissues and assigns to this metal an important rôle in photosynthesis. In molluscan bloods Mn may appear to take the place of Cu. However, its function, if not its occurrence, is rather problematical, and it does not take rank in prominence with the other two. Some reasons will be advanced, based on the present work, for believing that iron and copper, if not the only two elements possible, are nevertheless the two elements which are, chemically, especially well adapted to the rôle of oxidation catalysts.

One method of approach to the problem of biological oxidations is the measurement of respiration in organisms. Another is the attempt to duplicate, as far as possible, the conditions existing in the cell by means of inorganic chemical systems where the conditions may be controlled. The first method has led to the conclusion that iron, and to a lesser extent copper, is the catalytically active substance. The second method is more apt to furnish data regarding the mechanism of the reactions involved and is the one used in the present investigation.

II

In order to duplicate the essential conditions in living systems it is necessary to have an oxygen-rich substance (a peroxide), a catalyst, and an easily oxidizable substance. Ray (1923-24) has proceeded according to this principle and has investigated the system iron-hydrogen peroxide-unsaturated fatty acids from the point of view of

the effect of anesthetics. It was thought best here to use a combination which reacted quite rapidly, and therefore pyrogallol was used as the oxidizable substance. One of the end products of the reaction is carbon dioxide, the rate of production and amount of which may be accurately measured.

The experimental measurements were made with an Osterhout respiration machine the principles of which have been described in an earlier paper (Cook, 1925-26). Briefly, there is a closed system of tubes through which air is forced by a pump, from a reaction chamber to a tube containing an indicator (phenol-sulfonphthalein) and thence by another route to the reaction chamber again. The indicator is decolorized by the carbon dioxide and the color is restored by switching the current of air through a U-tube containing sodium hydroxide.

In carrying out the present series of experiments a definite amount of pyrogallol was dissolved in water in a large test tube (the reaction chamber) the tube was placed in its proper position in the circuit of the machine, and the carbon dioxide present in the solution was cleared out. Then through a separatory funnel a mixture of the metal salt and hydrogen peroxide was run into the reaction chamber. These two constituents were mixed *immediately* before being run in so that there might be as little reaction between them as possible before striking the pyrogallol solution the error here involved is entirely negligible because the reaction between the metal and the peroxide is relatively slow. Then the machine was started and the carbon dioxide produced was measured practically from the start of the reaction.

In order that there might be no possibility of the walls of the container exercising a catalytic effect, or otherwise disturbing the reaction, the inside of the reaction chamber was coated with paraffin and the coating renewed frequently. Furthermore, the indicator solution was replaced after every experiment in order to guard against any contamination by volatile organic acids which might be produced during the oxidation of the pyrogallol. Finally the reaction chamber was placed in a water bath and the temperature was kept uniformly at 25°C. in all the experiments here reported.

Unless the concentration of the reactants was purposely varied the mixtures were made up by dissolving 0.1 gm. of pyrogallol in 44 cc. of water (distilled) to which was added 1 cc. of hydrogen peroxide and 10 cc. of the metal salt in the desired concentration. The hydrogen peroxide was all taken from the same bottle and retained its strength at approximate constancy throughout the entire series of experiments.

Since the hydrogen ion concentration of the medium affects the rate of oxidation of pyrogallol it was ascertained colorimetrically that a mixture of 0.1 gm. of pyrogallol and 1 cc. of the peroxide in 44 cc. of water has a pH of approximately 5. Since most of the metal salts here used have an acid reaction there is no doubt that all the present experiments were performed in an acid medium. The pyrogallol

will not absorb oxygen as rapidly in an acid as in an alkaline medium, but since the hydrogen ion concentration did not vary to any great extent, and always remained on the acid side of neutrality, the relative values obtained are not invalidated. It would of course be impossible to conduct experiments by the indicator method with an alkaline medium in the reaction chamber.

It is customary when using the indicator method with an organism to express the results as a rate curve based on the normal rate of respiration as 100 per cent. Such a procedure is impossible here since there is no "normal rate." In fact there is no production of carbon dioxide previous to the start of the reaction.³ It is necessary, therefore, to find some other method of expressing the results. This may be done by using the absolute amounts of carbon dioxide produced. Ray (1923-24) has developed this method with the assistance of E. J. Cohn and has calculated the actual quantity of the gas. Here it is not essential to know the actual quantities in mg. Arbitrary units are satisfactory since all the results are relative and may be compared with each other even if we do not know the exact amounts. If the same amount of sodium bicarbonate is always present in the indicator solution and the buffer standards always remain constant, then it will always take the same amount of carbon dioxide to decolorize the indicator. We may then take this amount as a unit and calculate the number of units produced in a given time, or the length of time to produce 1 unit. But we must remember that the reaction is also proceeding during the time that the color of the indicator is being restored and include this time. For example, if it takes 30 seconds to decolorize and 30 seconds to restore the color then 2 units of carbon dioxide are formed per minute. Or if in 10 minutes there are four periods of decolorization 2 minutes long and four periods of 30 seconds to restore the color, then 5 units will have been formed during the 10 minutes.

Using this method the total amount of carbon dioxide produced can be plotted against the time and an integral curve obtained. If the equation of this curve is known then the rate curve can be derived therefrom.

III

Since this investigation has been primarily on the effect of copper, this element will be treated in a separate section. Fig. 1 shows typical curves obtained with 1 cc. of hydrogen peroxide, 0.1 gm. of pyrogallol, and various concentrations of copper chloride, in 44 cc. of water. The curves represent the total amounts of carbon dioxide produced, plotted against time. The slope of such a curve at any

³ Pyrogallol is oxidized by hydrogen peroxide and metal salts separately at such a slow rate (if at all) that it cannot be detected by this method. Hence we may say for practical purposes that there is no production of carbon dioxide unless all three constituents are present.

point indicates the rate of production of carbon dioxide. It will be seen that with very dilute copper the rate is at first relatively rapid, but soon falls off until it approaches zero. With concentrated solutions the rate decreases continually, but since the earlier portion of the curves is most important it was not considered necessary to follow the course of the reaction entirely to completion. In fact to do so with concentrated copper solutions would require many hours if not

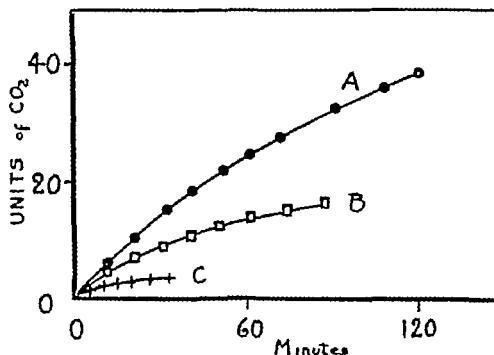


FIG. 1 The quantity of CO_2 produced by 0.1 gm. of pyrogallol 1 cc. of H_2O_2 , 44 cc of water and 10 cc. of

in Curve A, 0.0003 M CuCl_2

" B 0.0001 M,

" C, 0.00001 M, "

The ordinates represent arbitrary units of CO_2 and the abscissae minutes. Each curve is the average of three or more experiments.

days of continuous observation. The upper curves, therefore, are incomplete.

In order to compare and analyze curves of this sort the equations should be known. The attempt was made to fit the curves with the equations of a monomolecular and bimolecular chemical reaction. Although in some instances an approximate constant can be obtained the correspondence is not very close, and these equations have very little significance in the present connection. However

the curves can be fairly closely fitted by the general equation for the hyperbola $y = x/(a + bx)$. In all the experimental cases, when x/y is plotted against x the resulting figure is a straight line. There are individual deviations of slight extent but these are irregular in their occurrence and indicate that the experimental curves are fitted only approximately by the general equation $y = x/(a + bx)$. Even though the correspondence is only approximate, however, it is sufficiently close to permit the use of the equation in a purely empirical way for the comparison of the curves. In considering the present data the special equation may be used $a = t/(p + bt)$, where a is the amount of carbon dioxide produced after time, t , and p and b are constants.

The constants p and b must be determined for each different curve and when determined⁴ will be an index to the characteristics of the curve, within the same limits as were suggested with respect to the accuracy of the general equation as applied to these cases. The constants, with these reservations, may be used to compare the action of different concentrations of the reagents. To evaluate the constants the method of least squares was used⁵. In the experimental curves we are particularly interested in the total amount of carbon dioxide which will be formed if the reaction is allowed to run to completion, and the rate, specially the initial rate, of the production. These two quantities may be calculated from the formula $a = t/(p + bt)$ when the constants are known.

From an inspection of the curves it can be seen that as time proceeds the amount of carbon dioxide, called a , will approach a limiting value. Then, in the formula,

⁴ One source of error arises from the fact that different values for the constants will be obtained, depending on how much of the entire curve is used as a basis of calculation, and, as pointed out above, some of the experimental curves are incomplete. This error is accounted for, at least partially, by using only the first half of the complete curve, i.e., the curve up to the point where one-half of the total amount of the carbon dioxide has been produced. The total amount is observed in some cases and estimated in others.

⁵ The method of least squares is probably the most accurate method of determining the value of p and b , although any two points may be selected on a curve and the constants obtained by substituting observed values of a and t . For these curves the method outlined by Mellor (1909, p. 327) has been followed. Using the general equation $y = x/(a + bx)$ and a large number of points the following equations determine the constants

$$a = \frac{\sum (xy) \sum (x^2y^2) - \sum (x^2y) \sum (xy^2)}{\sum (y^2) \sum (x^2y^2) - (\sum (xy^2))^2}$$

and

$$b = \frac{\sum (xy) \sum (xy^2) - \sum (x^2y) \sum (y^2)}{(\sum (xy^2))^2 - \sum (x^2y^2) \sum (y^2)}.$$

allow t to become very large. Since p is constant, t , and also bt will become so large that p may be neglected. Then $a_{(lim)} = t/bt$. Cancelling t , $a_{(lim)} = 1/b$. The limiting value of a and the total amount of carbon dioxide (expressed in arbitrary units of course) will therefore equal $1/b$. This furnishes a convenient method for comparing the total amount of the action under different conditions.

In considering the rate of the activity we may deal with either a derivative or a tangent depending on whether we are considering an equation or a curve. With the experimental curves the tangents may be ascertained by means of instruments, and the rate curves then plotted if desired. The initial rate may be observed directly.

With the equation the procedure is different. If $a = t/(p + bt)$ then $da/dt = p/(p + bt)^2$. By substituting values for t the value of da/dt or the rate, may be plotted. Now let t become exceedingly small compared with p , and the differential equation approaches the value $da/dt_{(lim)} = p/p^2$ or $1/p$. Therefore $1/p$ represents the initial rate of the production of carbon dioxide.

Applying these methods it is possible to get information concerning the concentration effects of the various reagents. Fig 2 shows three curves obtained by plotting the values of $1/b$ against the concentrations of the reagents. Fig 3 shows similar curves for the initial rates, as obtained from the equation and also from the tangents to the experimental curves*.

When the concentration of the pyrogallol or of the copper is varied we find that the limiting value of a varies as a constant fractional power of the concentration, or $a_{(lim)} = C^k$. This relation has been found very frequently in biological work, and owing to its similarity to the adsorption isotherm it has caused many phenomena to be ascribed to adsorptive processes. The present case makes it plain that adsorption cannot always be called upon to explain every process where the effect is proportional to some fractional power of the concentration. For here we have nothing but a chemical system. Pyrogallol and hydrogen peroxide do not show colloidal properties, and it is very unlikely that copper chloride exists in anything but an ionic or molecular form. Hence if adsorption is responsible for the fractional

* The figures obtained by these two methods are almost identical in their general character the differences being due to the fact that different units are employed in the calculations. This close correspondence between the two sets of curves is evidence of the soundness of the method of calculation used in determining $1/p$. The values calculated for $1/b$ may also be accepted with confidence, since the manner of derivation is the same.

power relation then it must be in a sense very different from that commonly employed

With hydrogen peroxide a curious relation comes to light When its concentration is varied and the logarithm of the concentration is

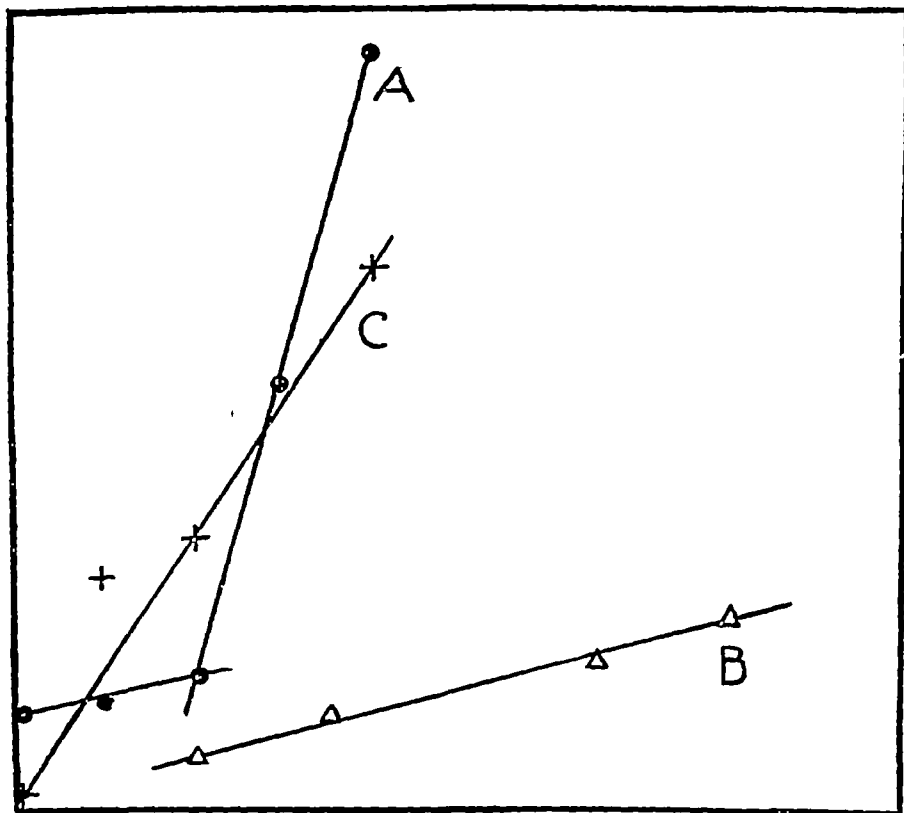


FIG 2 Effect of concentration on the total amount of CO_2 produced In all cases the ordinate is the logarithm of the amount of CO_2 as expressed by means of the logarithm of $1/b_{(11m)}$ (see text) The abscissa is

in Curve A, the logarithm of the concentration of CuCl_2 ,

" B, the logarithm of the concentration of H_2O_2 — the logarithm of the amount of CO_2 ,

" C, the logarithm of the concentration of the pyrogallol

plotted against the ratio of the logarithm of the concentration to the logarithm of the effect a straight line is obtained Expressed as an equation $\log a = \log C / (m + n \log C)$ That this relation has been

previously observed may be shown by four instances where data from Snapper (1912), Dreyer and Walker (1914), Nothmann Zuckerkandl (1912), and Plavec (1900), when plotted, give a straight line under similar conditions. However, the relation is unusual and no interpretation is available at present.

Fig 2, Curve A, shows the effect of varying the concentration of

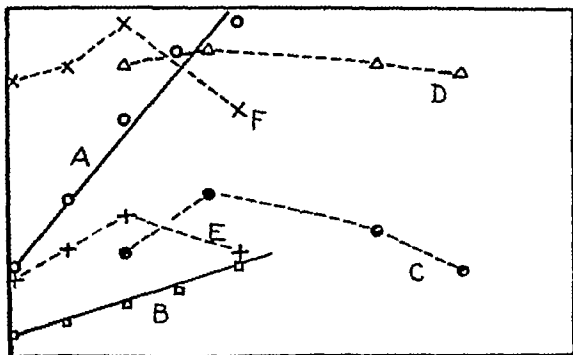


FIG 3 Effect of concentration on the initial rate of reaction In all cases the abscissa is the logarithm of the concentration. The ordinate in Curve A is the value of $1/p_{(lim)}$ for CuCl_2

In Curve B the value of tangents to the beginning of experimental curves of CuCl_2

- ' C the value of $1/p_{(lim)}$ for H_2O_2
- ' D, the value of tangents with H_2O_2 ,
- E, the value of $1/p_{(lim)}$ for pyrogallol,
- " F, the value of tangents with pyrogallol.

copper It will be noticed that while in general the law $a_{(lim)} = C^k$ is obeyed, still, at 0.0001 M, there is a distinct break and the exponent k is different on each side This is a situation which exists quite frequently in biological data. It might be maintained that at this critical concentration some change of phase relations occurs, such as the appearance of a new hydrate, dissociation product, or the like,

which would suddenly alter the effective concentration of the copper. While it is impossible to offer an adequate and detailed explanation of this phenomenon, nevertheless certain interesting possibilities are suggested, particularly with regard to phase relations in biological experiments.

Fig 3, Curve A, shows the effect of changing the concentration of copper on the initial rate of oxidation. Here the relation is da/dt (initial) = $k \log C$ or \tan (initial) = $k \log C$. The lines are not perfectly straight, indicating some deviation from the simple law, but the fit is close enough to show that there is a continuous and definite variation of initial rate with concentration. On the other hand, there is no such clear-cut variation with the pyrogallol or peroxide. The variation, if any, is slight and seems to pass through a maximum. If three points can be said to determine a straight line in such a case then the relation is the same as that with copper, *i e*, da/dt (initial) = $k \log C$. But here there is a break where k changes from plus to minus. If the data cannot be said to warrant such a conclusion then the initial rate must be considered independent of the concentration and the apparent linear arrangement of the points purely fortuitous.

Whatever the interpretation of the data, the difference between copper and the other two reagents is striking. It suggests that the mode of action of copper is unique, and that it undergoes changes, or takes part in reactions, in which the other two constituents are not involved.

IV

After copper the first metal investigated was iron. With hydrogen peroxide and pyrogallol it causes a rapid evolution of carbon dioxide. Contrary to what might have been expected, iron was but slightly more effective than copper. This doubtless is due, however, to the conditions existing in this particular system. Under other circumstances iron is a much more effective oxidation catalyst than copper. At the same time it should be borne in mind that as a toxic agent copper is far more powerful than iron.

The form of the time curve with iron was substantially the same as that with copper. The concentration effect was not investigated. One phenomenon worth mentioning appeared in connection with the

experiments on iron. If several experiments were performed, using the same concentrations of all reagents, each experiment showed a larger production of carbon dioxide than the one preceding. This rendered it almost impossible to duplicate an experimental result. When the inside of the reaction chamber was coated with paraffin and the paraffin renewed occasionally, this difficulty vanished. The results became reproducible. Such a situation indicates that part of the iron was adsorbed or deposited on the glass wall of the tube and influenced subsequent reactions. No deposition of iron took place

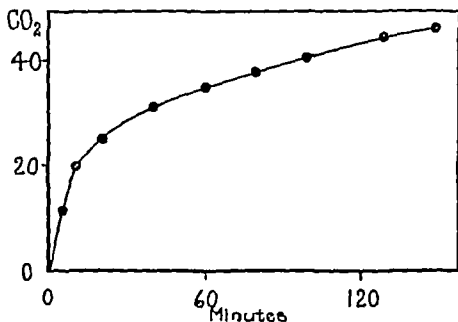


FIG. 4 Curve (average of three experiments) with AuCl_3 , 0.01 M. The ordinate is the amount of CO_2 and the abscissa is time in minutes.

on the paraffin. Although no perceptible influence was ever exercised by the walls of the tube in the experiments on copper, paraffin was used as a safeguard.⁷

Several experiments were performed with silver nitrate and gold chloride. The action of these two elements being very similar only one, gold, will be discussed in detail. When the reaction with gold chloride starts, the production of carbon dioxide is relatively rapid (although not quite so rapid, for equivalent concentration, as with

⁷ There are numerous instances recorded concerning the effect of the glass wall of the containing vessel on chemical reactions.

iron or copper) This period of great activity is followed by a much longer period of slight activity (see Fig 4) The curve rises quite steeply for about 10 minutes, at the end of which time the rise becomes much more gradual It appears as if there were two distinct curves, the first ending after about 10 minutes and merging into the second which continues for at least 2 hours Examining the reagents it is found that the gold, which is introduced as a clear, yellow, solution of gold chloride, has precipitated as purple colloidal gold If the reaction is followed in a test-tube it is observed that the precipitation begins after about 5 minutes and is apparently complete at 10 minutes This precipitation corresponds in time to the sudden falling off in the rate of oxidation Therefore the conclusion seems evident that the gold in solution catalyzes the oxidation of the pyrogallol to a marked extent, whereas the precipitated gold acts as a far less efficient catalyzer if indeed it catalyzes the reaction at all This matter will be discussed further in the next section

Silver precipitates in a manner almost identical with that of gold, and the same considerations apply as with the latter metal

Of several other metals none had a very marked effect Cobalt and manganese caused a very slight production of carbon dioxide They were each about as effective at a concentration of 0.01 M as copper at 0.00001 M, in other words, copper is about one thousand times as powerful a catalyst Magnesium, mercury, cadmium, zinc, tin, and nickel had no detectable effect whatever Hydrogen, in hydrochloric acid, was likewise without effect The metals investigated may therefore be arranged in the following manner

Group I *Catalysts* (in order of effectiveness), Fe, Cu, Au, Ag, Co, Mn

Group II *Non-Catalysts*, Mg, Hg, Cd, Zn, Sn, Ni, H

The theoretical significance of this grouping will be discussed later

v

When we come to consider the probable mechanism of this oxidation system two facts stand out as of primary importance

1 All the experiments were performed in an acid medium Hopkins (1925) has found in studying the mechanism of oxidation by means of glutathione that there is a marked difference in the course of the

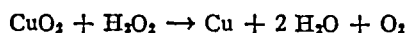
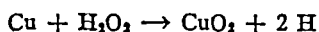
reaction depending on whether the medium is acid or alkaline, and Meyerhof (1923) maintains that oxygen transfer by sulfhydryl groups occurs only in acid and best at pH 3 to 5 (On catalysis by Fe in this connection, cf Harrison, 1924) These and other instances make it apparent that a change from an acid to an alkaline medium may profoundly alter the mechanism of an oxidation Therefore it should be borne in mind in the discussion of the present data that all conclusions apply to experiments performed in an acid solution and that very different results might be obtained with experiments performed in an alkaline solution

2 The experimental curves do not follow the course of a monomolecular or of a bimolecular reaction Since the possibility of a reaction of a higher order is remote, it is apparent that we have here a complex of reactions of different types To construct a hypothetical arrangement which would duplicate all the experimental data would be very difficult, but a simple system can be set up which will give a general representation of what is found experimentally

Let us consider the action of copper There are two distinct phases to the oxidation of pyrogallol by this metal First, the hydrogen peroxide must be decomposed in order to liberate the oxygen, and second, the oxygen must combine with the pyrogallol to produce carbon dioxide and other substances Considerable research has been performed on the problem of the decomposition of hydrogen peroxide by metals Bredig and Ikeda (1901) studied the decomposition of peroxide by platinum and considered that the action was due to the formation and reduction of an oxide of platinum Oliveri Mandalà (1920) found that a similar catalytic effect is produced by iridium, as had Bredig and Fortner (1904) with palladium Berthelot (1901) found that silver forms a peroxide and afterward a superoxide with hydrogen peroxide, both of which decompose and liberate oxygen Baeyer and Villiger (1901) started with the oxide Ag_2O and produced free oxygen with hydrogen peroxide Colloidal silver was precipitated during the reaction Hedges and Myers (1924) studied a periodic decomposition of hydrogen peroxide by silver, platinum, gold, and the enzyme catalase These authors do not believe in the formation of an intermediate oxide.

In connection with the problem of "promoter action" the effect of

copper and iron, as well as other substances, has been extensively investigated Bray and Livingston (1923) worked with bromine Their opinion is that the hydrogen peroxide is decomposed by the bromine molecule which is thereby ionized and which is later restored to the molecular state The ion is therefore the intermediate product Von Bertalan (1920)⁸ says "Die Zersetzung des Wasserstoffperoxyds durch Eisenionen verläuft als eine typische monomolare katalytische Reaktion" He considers the ion as the effective catalyst Mummery (1913)⁹ says "The catalytic decomposition of hydrogen dioxide by iron salts may be ascribed to the formation of higher perhydrols which are to be regarded as derivatives of hydrogen trioxide" Bohnson (1921) says that with iron the intermediate product is a hydrated ferric peroxide or "ferric acid" It is of interest that his data relating concentration to velocity constant (he finds the reaction to be of the first order) show breaks in continuity of very much the same sort as the break in Fig 2, Curve A, where copper is the catalyst Both ferric chloride and ferric sulfate exhibit this phenomenon Bohnson also found that the specific reaction velocity decreases with time owing to the hydrolysis of the catalyst, and that free acid retards the action Bohnson and Robertson (1923) conclude that the reaction, with iron, is an oxidation of the ferric ion, Fe, to a peroxide, FeO₄, and subsequent reduction to the ionic form They base their opinion on thermodynamic reasoning Goard and Rideal (1924) state that the intermediate compound varies according to the acidity of the solution, that in neutral solution it is Fe₂O₆ and in acid solution it is H₂FeO₄ Similarly, Spitalsky and Petin (1924) ascribe the activity of iron to intermediate products which vary in composition with the hydrogen ion concentration Robertson (1925) has summed up the discussion and has given an explanation of the so called promoter action of metals For the simple combination of copper and hydrogen peroxide he suggests the following equations



⁸ von Bertalan (1920), p 328

⁹ Mummery (1913), p 889

The CuO_2 is copper peroxide and when in acid solution may exist as H_2CuO_2

In view of the above accumulation of opinion and evidence it seems permissible to accept Robertson's equations as the basis for the first step in the oxidation of pyrogallol and to emphasize the following three points

- 1 The metal functions in the ionic form
- 2 There is always an intermediate product formed
- 3 This intermediate product is a peroxide

This system applies when there is simply a metal and hydrogen peroxide present. When an oxidizable substance is added it is necessary to alter the supposed course of the reaction. The fact that hydrogen peroxide will color guaiac in the presence of a metal salt has long been known. A recent study is that of Aloy and Valdivié (1923) on copper. Karczag (1921) has investigated the oxidation of dyestuffs by metal salts and hydrogen peroxide. Another investigation bearing on the present problem is that of Bredig and Ikeda (1901), who found that many organic substances, including pyrogallol, "poisoned" the reaction between the metal and the peroxide.

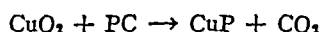
When an organic substance such as pyrogallol is oxidized by means of this system one of two things must happen. Either the oxygen which is liberated from the hydrogen peroxide unites with the pyrogallol, or the oxygen is transferred directly from the metallic peroxide without any separate existence. The latter supposition is more probable and is entirely in line with current theories of oxidation. If we accept it then we must notice two facts

- 1 The second stage of Robertson's reactions is altered because the oxygen in the CuO_2 does not escape as molecular oxygen but is transferred directly to the pyrogallol.

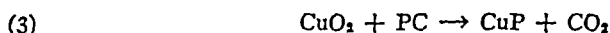
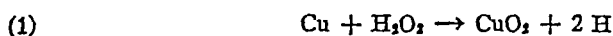
- 2 If this transfer released the ionic copper in its original state then the copper could go through the same cycle again and the reaction would continue until either the pyrogallol or the hydrogen peroxide was exhausted. But we know from experiments with low concentrations of copper that this is not the case. For instance, if we use 0.00001 M copper the production of carbon dioxide soon stops. If we then add more peroxide or pyrogallol there is no effect, but if we add more copper the oxidation begins again. This fact demonstrates

conclusively that the copper is steadily removed from the active state. The removal may take place in various ways but the simplest assumption is that the metal is bound up irreversibly with the pyrogallol. That some such process occurs is suggested by the results of Bredig and Ikeda (1901) on the "poisoning" effect of pyrogallol.

Let us call pyrogallol PC. Then



The carbon dioxide is measured and the inactive, or bound, copper remains in the solution. But we must complete the original equation. This we may do and write the entire scheme



Of course this scheme may differ in detail from what actually takes place. The real reactions are doubtless more complicated than is indicated here. But it is possible to go a certain distance toward duplicating the experimental results by means of the theoretical equations. Considering reactions (1) and (2) it is evident that there is a bimolecular reaction between the copper and the hydrogen peroxide. But there are two molecules of hydrogen peroxide decomposed for every molecule of copper peroxide formed, owing to the reduction of the second molecule of hydrogen peroxide by the free hydrogen. Using the ordinary notation, if a is the initial concentration of copper, b the initial concentration of the hydrogen peroxide, and y the amount of copper peroxide formed after time t , then the rate of the reaction is proportional to the copper and hydrogen peroxide present. Of the latter, part is used in step (1) to form CuO_2 and H , and part in step (2) to form H_2O . These two parts are equal. Then we may write $dy/dt = K_1(a - y)(b - 2y)$. This equation may be integrated and expressed in the following form

$$y = \frac{a b (1 - e^{K_1(a-b)t})}{b - 2a e^{K_1(a-b)t}}$$

If x is the amount of carbon dioxide formed after time t , and c is the concentration of the pyrogallol, then x is proportional to the amount of pyrogallol and the amount of CuO_2 (or y) present after t units of time. This amount of CuO_2 is equal to the difference between the quantity produced from a and b and the quantity which has been transformed into x . Then $dx/dt = K_1(y - x)(c - x)$, or

$$x = \frac{y c (1 - e^{K_1(y-c)t})}{c - y e^{K_1(y-c)t}}$$

Since y is a variable quantity its value must be determined for every value of t and substituted in the above equation

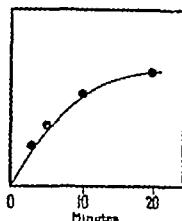


Fig 5 The solid line represents a typical experimental curve (CuCl_2 0.001 M H_2O_2 1 cc., pyrogallol 0.001 gm.) The points are obtained by means of the calculation outlined in the text whereby two bimolecular reactions are assumed. In this case $a = 0.5$, $b = 2$, $c = 0.03$, $K_1 = 0.2$ and $K_2 = 0.2$.

By the use of these equations the experimental curves may be approximately duplicated. Fig 5 shows an example of an experimental curve selected at random and fairly closely fitted. The fit could be made much closer by more exact selection of the constants. This method of curve duplication does not afford absolute proof that the equations underlying the calculations are precisely those which underlie the experimental results. However they must be fairly close to the truth. That the actual state of affairs is more complicated than has been suggested here is shown by one fact. In the calculated system the effect varies directly with the concentration, not as a fractional power thereof. In order to bring the calculated results into accordance with the experimental results it would be necessary

to assume further complications such as side reactions, opposing reactions, *etc* But the experimental data do not clearly indicate the precise nature of these additional reactions

The definite conclusions which may be drawn are as follows The oxidation of pyrogallol is due to the decomposition of the hydrogen peroxide by the copper ion, with the subsequent peroxidation of the copper The copper peroxide then transfers its oxygen to the pyrogallol which is broken down into carbon dioxide and other products, including some form of combined copper¹⁰

This system coincides with the Engler theory of respiration, in which there is an autoxidizable substance, a peroxide, and a catalyst Atmospheric oxygen is absorbed and transferred by means of the peroxidase (catalyst) through the peroxide from the autoxidizable substance to the substance which is eventually to be oxidized Here, of course, we start with a peroxide but the principle is the same Wieland (1921) takes the view that hydrogen, not oxygen, is transferred, but he states that with metals and hydrogen peroxide metallic peroxides

¹⁰ The question of the atmospheric oxidation of pyrogallol and the nature of the products when the reaction is catalyzed by a metal has received attention from chemists The two best known oxidation products are carbon dioxide and purpurogallin The latter is specially likely to appear when iron is present Salts of iron as well as of other metals may combine directly with pyrogallol, the reaction usually showing a characteristic color Thus Jacquemin (1873) found that ferrous sulfate gives a blue color and ferric sulfate a red one

de Clermont and Chautard (1882) got purpurogallin from pyrogallol in acid solution with AgNO_3 and KMnO_4 Wolff (1908) used ferrocyanide with hydrogen peroxide and obtained purpurogallin Smirnow (1925) determined the effect of peroxidases by measuring the formation of purpurogallin, and found that manganese and iron catalyze the reaction although he does not consider that these two metals are necessary to a peroxidase He quotes André (André, G, 1924, *Chimie végétale*, 1, 210) to the effect that pyrogallol oxidizes according to the equation $4 \text{C}_6\text{H}_6\text{O}_3 + 9 \text{O} \rightarrow \text{C}_{20}\text{H}_{16}\text{O}_9 + 4 \text{CO}_2 + 4 \text{H}_2\text{O}$ where $\text{C}_{20}\text{H}_{16}\text{O}_9$ represents purpurogallin He also quotes Willstätter and Stoll (Willstätter, R, and Stoll, A., 1918, *Ann Chem*, cdxvi, 62), who state that $2 \text{C}_6\text{H}_6\text{O}_3 + \text{O}_3 \rightarrow \text{C}_{11}\text{H}_8\text{O}_6 + \text{CO}_2 + \text{H}_2\text{O}$ The latter formula ($\text{C}_{11}\text{H}_8\text{O}_6$) is given as the correct formula for purpurogallin in Beilstein, F, 1923, *Handbuch der organischen Chemie*, 4th edition, vi, 1072 In the experiments here recorded the concentrations of the reagents were too low for noticeable quantities of purpurogallin to be produced

are formed. He therefore agrees in essentials with the intermediate peroxide theory.

VI

Throughout the preceding discussion the assumption has been made that the metals act in the ionic form. This assumption is based on the results of Bohmson, Robertson, etc., according to whom the metals (in decomposing hydrogen peroxide) take part in ordinary chemical reactions. There is no reason to suppose that there is any change in the state of iron or copper when pyrogallol is present. Nevertheless there is evidence to show that certain metals in the colloidal state can also decompose hydrogen peroxide. Certain metals, furthermore, are precipitated in the colloidal form by pyrogallol. Bredig and Reinders (1901) decomposed hydrogen peroxide with colloidal gold, as did Galecki (1925). Bredig and Ikeda (1901) used colloidal platinum, Duclaux (1923) used colloidal iron, and Zengheis and Papaconstantinos (1920) used colloidal rhodium. As to the effect of pyrogallol, in addition to calling attention to the reduction of silver bromide by pyrogallol in photography, reference may be made to Garbowski (1903), who precipitated gold, platinum, and silver, and to Henrich (1903), who precipitated gold, platinum, silver, and mercury with pyrogallol.

We have a very clear example of the different effect of ionic and colloidal metal in the experiments performed on gold which were outlined in a previous section. In these experiments we can watch the rate of oxidation change while the colloidal gold is being precipitated. The first few readings are obtained with ionic gold and show a rapid action. After the gold has been precipitated the action still continues but at a greatly reduced rate. There exist at this point two possibilities: either the gold is all precipitated and the slow action is due to the colloidal metal, or not all the gold is precipitated, the colloidal metal has no effect, and the slow rate is due to a very much reduced quantity of the ionic metal. To throw light on this matter a standard quantity of gold chloride was added in a test tube to the usual amount of hydrogen peroxide and pyrogallol and after the gold had been precipitated the entire mixture was dialyzed for a week.

Then the colloidal gold was recovered, added to more hydrogen peroxide, and pyrogallol and the resulting action tested in the respiration machine. There was no detectable production of carbon dioxide. The conclusion follows that colloidal gold does not catalyze the oxidation of pyrogallol in measurable quantities, despite the fact that it decomposes hydrogen peroxide.

The question arises why colloidal gold does not oxidize pyrogallol. It will be remembered that to account for the action of copper it was assumed that the copper forms a peroxide which carries the oxygen from the hydrogen peroxide to the pyrogallol. It does not liberate molecular oxygen which then, of itself, combines with the pyrogallol. The formation of the intermediate peroxide is essential. Now it is clear that while ionic gold can react to produce a gold peroxide, the metal in the colloidal form cannot do so. Therefore as the gold in solution becomes aggregated in solid particles the concentration of the effective gold is reduced. That the ions are not completely eliminated and that all the gold is not changed into the colloidal form (as is demonstrated by the fact that some oxidation persists) is probably due to the fact that the solution is acid. That the degree of dispersion, size of particles, and general properties of colloidal metals are dependent to a large degree on the acidity of the medium has been shown by numerous investigators. The presence of the pyrogallol doubtless has considerable influence and may tend to prevent the complete precipitation of the gold in solution.

Obviously the mode of action of the metal in the colloidal state is very different from the mode of action in the ionic form. As the present case shows, ionic gold will catalyze the oxidation of pyrogallol while colloidal gold will not. But the latter will decompose hydrogen peroxide as well as the former. The decomposition, however, must be due to an entirely different mechanism in the two cases. The intermediate peroxide theory, which is very satisfactory with the metal in solution, offers no explanation whatever for the action of the colloidal metal since the colloid cannot be expected to enter into simple stoichiometric relations with the other constituents, including the formation of metallic peroxides. If, as has been assumed, the oxidation of pyrogallol depends on the presence of such peroxides, it is quite evident

why colloidal gold can decompose hydrogen peroxide but fails to oxidize pyrogallol.

VII.

We are now in a position to make further comparison between the several metals which have been tested. A division has already been made between the effective catalysts, copper, iron, gold, silver, cobalt and manganese, and the non-effective metals which include all the others tried. Among the first four there are marked differences depending on the ease with which the colloidal metal is precipitated. As a check on this point test tube experiments were made which showed that gold and silver precipitated very easily, copper very slowly (a matter of days), and iron not at all. Iron is therefore the most powerful catalyst because it readily forms a peroxide and has the least tendency to be precipitated in the colloidal form by hydrogen peroxide and pyrogallol. Gold and silver are effective catalysts (though not so effective as iron and copper), but are prevented from exercising this function because they are so easily precipitated. Mercury, zinc, and the others, are ineffective because they do not form intermediate peroxides.

Herein may lie the reason why iron, and secondarily copper, are almost universally found in living cells as the metals which catalyze respiration. The assumption is legitimate that the cell contains organic peroxides similar to hydrogen peroxide and reducing (easily oxidizable) substances like pyrogallol. If this is true and there is no strong evidence against it, then iron and copper are probably the only two metals which could effectively catalyze the oxidations in the cell. For metals like cobalt and manganese would be too weak in their action, mercury, zinc, etc., would not carry oxygen at all, and gold or silver would pass immediately into the ineffective colloidal state. Therefore iron and copper best fulfil the requirements for a metallic catalyst.

One final theoretical aspect of this question deserves brief consideration. If we examine the periodic table of the elements as revised to conform to the most recent ideas in physical chemistry, we find that all the active elements here investigated, *i.e.* iron, copper,

gold, silver, cobalt, and manganese will be found close together in the table. Below is a reproduction of a portion of the table showing the arrangement of electrons in the atoms of the elements (according to Langmuir), found in Taylor's Treatise on physical chemistry (1924) ¹¹

	IIIa	IIIb	IVa
Group VII	Mn	—	—
Group VIII	Fe	Ru	Os
	Co	Rh	Ir
	Ni	Pd	Pt
Group I	Cu	Ag	Au
Group II	Zn	Cd	Hg

The groups refer to the standard groups in the periodic table (The numerals at the head of the columns refer to the number of shells of electrons). Thus we see that Group I, which includes copper, silver, and gold, has an outer shell of one electron, and Group VIII has an outer shell which lacks completeness by one electron. It is in these two groups that the effective elements Fe, Cu, Ag, Au, and Ru, Rh, Pd, Os, Ir, and Pt, which are probably also effective, occur. The only exceptions are manganese which is in Group VII, and nickel which is in Group VIII but is not effective. In general the situation is striking. Those elements which are effective oxidation catalysts have their outer shell either composed of just one electron or lacking just one electron. Furthermore, as the number of shells increases the greater becomes the tendency for the element to pass into the colloidal state with hydrogen peroxide and pyrogallol. It is impossible to draw any conclusions at present from these facts, but they are very suggestive and indicate the possibility of future theoretical developments.

SUMMARY

1 When iron and copper are allowed to act on hydrogen peroxide and pyrogallol, enough carbon dioxide is produced to be readily measured

¹¹ Taylor (1924), p. 1062

2 The curve of the production of carbon dioxide may be fitted by an empirical equation, by the use of which the initial rate and the total amount of the oxidation may be determined

3 The effect of the concentration of the reagents is different in each case, the effect varying as a fractional power of the copper and pyrogallol concentrations and as a logarithmic function of the hydrogen peroxide concentration

4 When gold or silver is used the rate changes suddenly during the course of the reaction due to the precipitation of colloidal metal.

5 Mercury, cadmium, zinc, tin, and some other metals have no effect.

6 A theoretical set of equations is assumed to account for the action of the metals

7 The metals are assumed to act by means of the formation of intermediate peroxides

8 Experiments on the action of gold indicate that the metals are active in the ionic and not in the colloidal state

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AMPHOTERIC BEHAVIOR OF COMPLEX SYSTEMS

I THEORETICAL.*

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The attempts to explain a part of the behavior of living cells or tissues on the assumption that they act as simple ampholytes, while fairly satisfactory and suggestive, must be considered only as a first suggestion. Those who employ this concept admit that living cells are more complicated systems than simple proteins, so that clear-cut results from studying them from such a point of view are not to be expected. Certain recent researches cannot, however, be easily correlated on the basis of mere lack of "clear-cutness." For example, Robbins (1), from the staining reactions and water absorption of potato tuber, has shown that it acts as an ampholyte with an isoelectric point at a pH of about 6. However, Cohn, Gross, and Johnson (2) have found that the typical potato protein, tuberin, obtained by acid precipitation of potato juice, has an isoelectric point at a pH of about 4.

Winslow, Falk, and Caulfield (3) have studied the electrophoretic behavior of the organism *Bacillus cereus* over a wide pH range, and while, in the main, the curve obtained may be explained on the basis of a simple Donnan equilibrium, there is a comparatively wide pH range through which such an explanation cannot hold.

Certain unicellular organisms seem to show little tendency to retain either acid or basic dye through a comparatively wide pH range, in place of through only a narrow range as might be expected to be characteristic of the isoelectric behavior of a simple ampholyte (4). Other organisms show no point where combination with one

* Contributions from the Gates Chemical Laboratory California Institute of Technology No 115

or the other type of dye does not seem to take place to a considerable extent

It is the purpose of this paper to examine the probable behavior of a system of two amphoteric substances between which mutual combination may take place under proper conditions, and to show that, by employing the considerations involved, it is much easier to explain much of the physical and chemical behavior of living tissues than it is by using the concept of a simple ampholyte. In the two following papers experimental evidence is adduced, from a study of certain simple systems of two ampholytes, in support of this idea. It may, however, be pointed out that living cells are by no means as simple as this above concept would seem to indicate. Its justification lies in the fact that, by sacrificing only very little of the simplicity of treatment which suffices for consideration of simple ampholytes, one gains greatly in comprehensiveness.

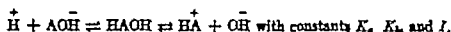
Concept of a Conjugate Protein

Consider an aqueous solution of two amphoteric substances, HAOH with ionization constants K_a and K_b and an isoelectric point at a hydrogen ion concentration I , and HBOH with corresponding constants K'_a and K'_b and an isoelectric point at I' . Suppose I is larger, i. e. more acid, than I' .

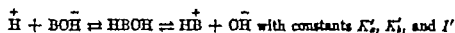
In solutions whose hydrogen ion concentration is appreciably greater than I both components will act as bases and tend to retain acids. When the hydrogen ion concentration is appreciably less than I' both components will act as acids and tend to retain bases. In solutions whose hydrogen ion concentration lies between I and I' , however, one component will act as a base and the other as an acid, and there will be a tendency toward mutual combination, resulting in a decreased retention of acid or basic reagent. This pH range, from I to I' , is the range of mutual combination, and the stability of the system, so far as its behavior as a distinct individual is concerned, depends among other things on the magnitude of this range. This might explain, for example, why certain conjugated proteins, such as lecithoproteins, though they are thought to exist, have not been definitely isolated, while others such as nucleoproteins or phosphoproteins can be easily obtained.

Apparent Isoelectric Behavior

In a system of two ampholytes we have the following equilibria



and



If HAOH is the more strongly acidic, there will be an isoelectric point at the pH at which

$$(A\bar{O}\bar{H}) + (B\bar{O}\bar{H}) = (H\bar{A}) + (H\bar{B})$$

The value of the hydrogen ion concentration corresponding to this point is obtained by expressing the above quantities in terms of $(\overset{+}{H})$ and the various constants and solving. We obtain

$$(\overset{+}{H}) = \sqrt{K_a \frac{K (HAOH) + K' (HBOH)}{K_b (HAOH) + K'_b (HBOH)}} \quad (1)$$

The isoelectric point is not exactly the point of maximum mutual combination. The latter point may be expected to be governed by the condition

$$(A\bar{O}\bar{H}) = (H\bar{B})$$

and will occur at a hydrogen ion concentration obtained from the following expression

$$(\overset{+}{H}) = \sqrt{K_a \frac{K (HAOH)}{K'_b (HBOH)}}$$

The two points, though not identical, will lie very close together, and the simple expression may be used for calculating the isoelectric point of the system.

Electrophoretic Behavior

From the amphoteric equilibria given above we can, by applying the mass-action law and differentiating with respect to the logarithm

of the hydroxide ion concentration, obtain the following four equations

$$\frac{d(\text{AOH}^-)}{d \ln(\text{OH}^-)} = \frac{K_a (\text{HAOH}) (\text{OH}^-)}{K_w} \quad (2)$$

$$\frac{d(\text{HA}^+)}{d \ln(\text{OH}^-)} = - \frac{K_b (\text{HAOH})}{(\text{OH}^-)} \quad (3)$$

$$\frac{d(\text{BOH}^-)}{d \ln(\text{OH}^-)} = \frac{K'_a (\text{HBOH}) (\text{OH}^-)}{K_w} \quad (4)$$

$$\frac{d(\text{HB}^+)}{d \ln(\text{OH}^-)} = - \frac{K'_b (\text{HBOH})}{(\text{OH}^-)} \quad (5)$$

from which

$$\frac{d(\text{AOH}^-)}{-d(\text{HB}^+)} = \frac{K_a (\text{HAOH}) (\text{OH}^-)^2}{K'_b (\text{HBOH}) K_w}$$

or, since

$$(\text{OH}^-) = K_w/(\text{H}^+),$$

$$d(\text{AOH}^-) = - \frac{K_a (\text{HAOH}) (\text{OH}^-)}{K'_b (\text{HBOH}) (\text{H}^+)} d(\text{HB}^+)$$

Either an increase in (AOH^-) or a decrease in (HB^+) will increase the resultant negative charge on the micella, and thus increase the velocity toward the anode in a constant electric field. At the isoelectric point, as the hydroxide ion concentration is increased, $\frac{d(\text{AOH}^-)}{d \ln(\text{OH}^-)}$ is of the same order of magnitude as $\frac{-d(\text{HB}^+)}{d \ln(\text{OH}^-)}$ but the former increases with increasing alkalinity while the latter decreases (equations (3) and (4)). Through the pH range I to I' , the value of $-d(\text{HA}^+)$

is small compared to that of $d(\text{AOH})$, and $d(\text{BOH})$ is small compared to $-d(\text{HB})^+$. As a result there will be a rather rapid increase in negative charge through a certain range, passing through the isoelectric point, until nearly all the HAOH is ionized to AOH , which ionization takes place increasingly rapidly as (OH) is increased. When such a condition is reached, the only possible significant increase in negative charge before the point I' is reached is from $-d(\text{HB})^+$, which has less and less effect on the magnitude of the charge as $(\text{HB})^+$ becomes smaller, i.e. as (OH) is increased (equation (4)). This means that through a certain pH range the negative charge will remain nearly constant. When I' is reached, however, we have $\frac{-d(\text{HB})^+}{d(\text{BOH})} = 1$, and from this point on $\frac{d(\text{BOH})}{d \ln(\text{OH})}$ is the predominant factor. Its value increases with (OH) , and the negative charge again begins to increase more rapidly, and continues until the HBOH is completely ionized. From this point on, since we are now on the alkaline side of the isoelectric points of both components and they are thus both in the same ionic state, we may expect the curve to be the same as would be predicted by the application of the Donnan equilibrium to a simple ampholyte.

A similar condition would prevail on the acid side of the isoelectric point of the system as the hydrogen ion concentration is increased.

DISCUSSION

It may be well to cite some of the observations which originally led to the more definite formulation of the concept of a mixed ampho-teric system.

In connection with certain bacteriological problems the staining reactions of a large number of organisms have been studied by the author (4). Certain of the typical curves are given in Fig 1.¹ Ab-

¹ Bacterial cells furnish a very satisfactory material to study. The individual cell as a system can be easily observed, thin smears can be obtained fairly free from debris, and equilibrium can be quickly reached. We found little difference in results between buffering for several minutes and for as long as 150 hours. Gern

scissæ are pH values and ordinates are arbitrary functions of the intensity of retained color. Values for the latter were obtained by repeated comparison of slides under the microscope, and are there-

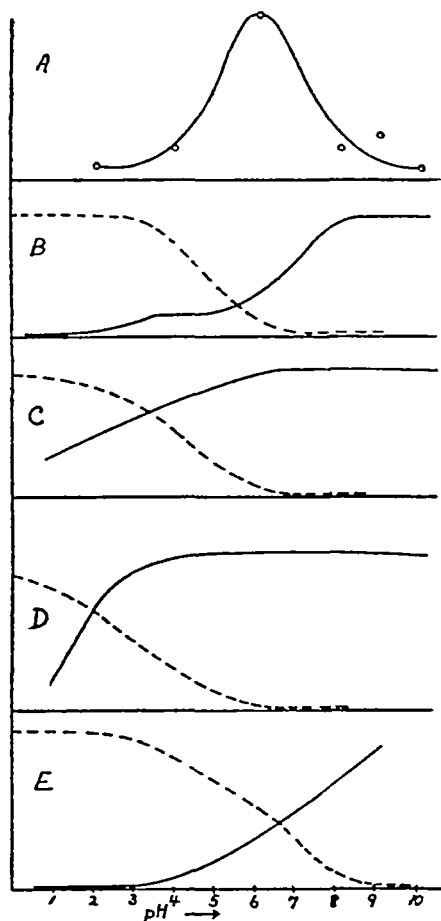


FIG 1 For explanation see text. Broken lines represent behavior toward acid fuchsin, unbroken lines (except Curve A) behavior toward gentian violet

gross, along the same line (6), states that hide powder in small quantities, unhardened by formaldehyde, reaches complete reversible equilibrium with acid solutions in 2 minutes. Our final technique consisted in preparing thin smears on microscope slides, staining with "carbol gentian violet" or "carbol acid fuchsin" solution, "fixing" by treatment with buffer solution, and finally decolorizing with acetone. Slides of any series were then repeatedly compared under a microscope

fore only qualitative. They are, however, comparative and the curves represent the behavior of the organism. The intensity plotting has been conservative and quantitative methods would, we feel sure, merely accentuate the contrast between the greater and smaller dye retention.

In Curve A of this figure the buffer ratios for the organism *Bacillus coli*, obtained by Falk and Shaughnessy (5) are plotted just above the color curve for the same organism (Curve B).² The results are suggestive, showing that the buffering power of this organism shows itself over a wide pH range with a maximum near the isoelectric point of the system as determined by minimum dye retention.

The curves in this figure are typical of classes of bacteria. A fair number of organisms have their point of least color retention around a pH of about 3 (Curve D), another large class has a corresponding point around a pH of 5 to 6 (Curve B), and there are a few intermediate (Curve C).

Other types of cells were studied, such as red blood cells from both human blood (Curve E) and sheep blood. In the case of the former the smears were made from whole blood and the isoelectric point of such a system lies a little below a pH of 7. The sheep cells used were washed cells and showed a corresponding point at a pH very near 7.

Such a system as *Bacillus coli* (Curve B) exhibits a range through which little dye is retained—either acid or basic—suggesting a considerable stability of the system as a chemical individual, while the system *Bacillus dysenteriae* Shiga (Curve C), even at its isoelectric point, still retains fairly strongly both acid and basic dyes, showing that there is still a fair concentration of both cation and anion in the system, and that either actual combination between these two is limited, or that the combination is comparatively unstable.

The condition of maximum combination between the two components of a system of two ampholytes need not mean a condition of much combination. When $(\text{AOH}) = (\text{HB})$, though this is the optimum condition for combination, the system may act as a majority

² In plotting Curve A the pH range given in the table by Falk and Shaughnessy is plotted at its lowest value, thus where they give the range 6-7 it is plotted as 6.

of ionogens and remain largely in ionic form. On the other hand it may act in a manner analogous to such salts as lead acetate, mercury salts, etc., and the two ions may almost entirely combine. The extent of this combination will determine the behavior toward anions and cations. In systems in which the two components are largely combined at the isoelectric point we may have behavior simulating a simple ampholyte in that there will be a pH range through which no appreciable combination with added cation or anion takes place.

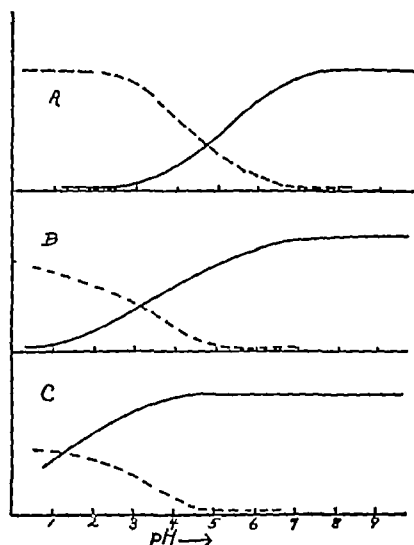


FIG. 2. Effect of oxidation on the behavior of *Bacillus typhosus* toward acid fuchsin (broken line) and gentian violet (unbroken line). Curve A—original organism, Curve B treated with N/50 iodine, Curve C treated with N/50 potassium dichromate.

On the other hand, we may have systems in which even at the isoelectric point there is still a fair concentration of both (AOH) and (HB^+) and thus there will be no point at which added anions and cations will not be appreciably bound.

There are at least two ways in which the isoelectric point of a mixed system or of a simple ampholyte may be changed. The system may be transformed into a new mixed system either by altering the relative amounts of the components or by adding another component,

or the acid or basic properties may be altered by oxidation or reduction

An example of the first effect is reported by Gerngross (7) who found that the electrophoretic isoelectric point of gelatin was changed from a pH of 4.75 to 4.3 by treatment with formaldehyde

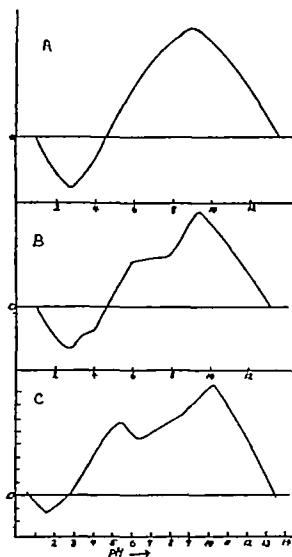


FIG 3

Change in isoelectric point by oxidation has been studied by the author (4), and certain results are shown in Fig 2. The magnitude of the change depends on the degree of oxidation. A mild oxidizing agent such as iodine (Curve B) renders the system more acidic than it was originally (Curve A), but such an oxidizing agent as potassium

bichromate produces a distinctly greater shift in the same direction (Curve C) ³

While reducing agents do not seem to have any effect on the original organism, the effect produced by oxidation has been repeatedly reversed by treatment with stannous chloride. This behavior is, of course, analogous to the behavior of practically all substances in the effect of oxidation or reduction on acid strength.

An interesting application of the concept of a mixed system is the electrophoretic behavior of the organism *Bacillus cereus* as worked out by Winslow, Falk, and Caulfield (3) and by Winslow and Shaughnessy (8). Their results are roughly represented by Curve C of Fig. 3. Abscissæ are pH values and ordinates are migration velocities in an electric field. These are measured toward the anode on that portion of the curve above the zero line and toward the cathode below this line. Curves A and B are both theoretical. Curve A represents the theoretical electrophoretic behavior of a micella composed of a single ampholyte to which the Donnan equilibrium applies. Curve B represents the theoretical electrophoretic behavior of a system of two ampholytes as worked out above. The intersection of the curves with the zero line represents the isopotential point.

In all the cases mentioned above the concept of a mixed system of ampholytes, so simple as to contain only two components, offers a much more obvious explanation of the experimental facts than the concept of a simple ampholyte.

SUMMARY

The amphoteric behavior of a system of two amphoteric components is theoretically examined, and this is shown to correspond more nearly with certain of the physical and chemical behaviors of living tissues than does the concept of a simple ampholyte.

³ Oxidizing agents were incorporated in the buffer solutions in N/50 concentrations ¹. A goodly number of oxidizing agents were studied, and, in general, they could be arranged in a series on the basis of the magnitude of their effect on the organisms, which series was roughly the same as arrangement on the basis of oxidizing potential. The increase in acid properties upon treatment with an oxidizing agent, illustrated in Fig. 2, was noted in all organisms studied.

It is a pleasure to acknowledge the criticism and suggestions of Dr A. L. Raymond in the preparation of the manuscript of this and the three following papers.

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AMPHOTERIC BEHAVIOR OF COMPLEX SYSTEMS

II. TITRATION OF SULFANILIC ACID-GLYCINE MIXTURES *

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(Accepted for publication, July 26 1926.)

The considerations presented in the preceding paper were developed as an attempt to explain results obtained in studying living cells (1) It is desirable to test them on much simpler systems Michaelis and Davidsohn (2), in some flocculation experiments, obtained data which support the above ideas in a qualitative way They state that, if two amphoteric colloids are mixed, a combination may precipitate out whose flocculation optimum lies between their respective isoelectric points. Thus with nucleic acid and denatured serum albumin, with isoelectric points of 2×10^{-1} and 4×10^{-2} respectively, there is a combination with optimum flocculation at a hydrogen ion concentration of 1.6×10^{-4}

It is the purpose of this and the following paper to test portions of the theory in a more quantitative manner, and in this paper the mutual action of two ampholytes, glycine and sulfanilic acid, is studied by means of titration over a wide pH range. The latter substance was chosen to correspond to the strongly acid nucleic acid in living matter That it is amphoteric, with a measurable basic ionization constant, is shown in the fourth paper of the series In fact it has an isoelectric point at a distinctly higher pH, 1.25, than that (0.7) of nucleic acid (2) The glycine used was a preparation from the Eastman Kodak Company, and the sulfanilic acid was a Merck c p grade, which was reprecipitated from alkaline solution by hydrochloric acid, washed, and dried

* Contributions from the Gates Chemical Laboratory, California Institute of Technology, No 116

Method.

An ordinary potentiometer set up sensitive to 0.25 millivolt, was employed. The hydrogen electrodes were of the type described by Bailey (3). A saturated calomel electrode (4) was used in connection with a saturated KCl bridge.

A series of solutions of hydrochloric acid and carbon dioxide-free sodium hydroxide was made up, each solution of definite known con-

TABLE I

HCl				NaOH			cOH
N	E.M.F.	pH	cH	N	E.M.F.	pH	
	mv				mv		
001	426	3.00	001	004	924.5	11.425	00266
003	399	2.533	00293	009	943	11.74	0055
006	380	2.217	00607	015	958	12.00	010
009	370	2.050	00891	025	968.5	12.175	015
012	363	1.933	0117	040	981.7	12.390	0246
018	353	1.760	0174	060	990.3	12.540	0347
025	345.5	1.625	0237	080	997.5	12.660	0458
040	333.5	1.425	0376	100	1001.5	12.730	0538
060	323	1.250	0562	130	1008.5	12.860	0720
090	313	1.083	0826	180	1014.8	12.960	0916
120	305	0.950	1122	250	1024	13.117	1310
150	300.5	0.875	1334	3511	1030	13.217	165
200	292.5	0.742	181				
250	287.8	0.663	217				
320	282	0.56	2754				

centration. Points on the titration curve were obtained by introducing 15 cc of one of these solutions into a small glass-stoppered bottle with 10 cc of a standard solution of the substance to be titrated, and the equilibrium hydrogen ion concentration of the resulting mixture determined. The difference between the normality of the acid or base diluted with 10 cc of water and that diluted with 10 cc of the glycine or the sulfanilic acid will give the amount neutralized. The original normality, N , is known and the latter can be obtained from the measured cH if we know the degree of ionization, α ; the normality is equal to cH/α , where α is the degree of ionization, which value

must be determined *potentiometrically* (5) Thus the number of mols, n , of the HCl or the NaOH neutralized by the glycine or sulfanilic acid is given respectively by the expressions

$$n = N - \frac{cH}{\alpha} \text{ for HCl and } n = N - \frac{cOH}{\alpha} \text{ for NaOH}$$

The only assumption involved is that at the same normality the acid

TABLE II

N HCl	S.M.P	pH	cH	n
	mg.			
320	285	0.617	2415	0.41
250	291.8	0.730	1862	0.395
200	298.5	0.842	1439	0.40
150	307.5	0.992	1019	0.390
120	316	1.133	0736	0.405
090	326.5	1.303	0492	0.375
060	344.5	1.608	02466	0.345
040	365.3	1.970	01072	0.291
025	388	2.350	00477	0.205
018	400.5	2.558	00277	0.152
012	414	2.800	00159	0.104
009	422.5	2.942	00114	0.0786
006	434	3.133	000736	0.0526
003	455.5	3.492	000322	0.0268
001	484	3.98	000105	0.009
0000	598	5.9	—	—
NaOH			cOH	
004	766	8.75	0000056	0.04
009	790	9.15	000014	0.09
015	806.5	9.43	000027	0.15
025	831.5	9.86	000073	0.249
0373	871.5	10.53	00034	0.368
040	895	10.933	00085	0.387
060	964	12.10	01259	0.40
080	981	12.383	02415	0.397
100	990.5	12.542	03483	0.401
130	1000.5	12.708	05105	0.397
180	1009.5	12.875	0750	0.398
.250	1019	13.033	1079	0.405
3511	1028	13.183	1524	0.40

or alkali ionizes to the same extent in the presence of the glycine or sulfanilic acid as it does when these are absent

Table I gives values of cH and cOH for various normalities of HCl and $NaOH$ at room temperature, $20-22^{\circ}C$ By plotting N

TABLE III

Sulfanilic acid				Glycine			
0.04 N		0.023 N		0.10 N		0.02 N	
pH	n	pH	n	pH	n	pH	n
				1.14	0.97	0.913	0.20
				1.483	0.864	1.033	0.19
				2.26	0.544	1.183	0.194
				2.56	0.372	1.408	0.186
				2.86	0.236	1.638	0.16
				3.033	0.178	2.01	0.15
				3.233	0.142	2.192	0.115
				3.37	0.086	2.467	0.086
				3.55	0.057	2.622	0.066
				3.85	0.0286	2.867	0.0464
						3.20	0.0237
						3.70	0.008
2.13	—	2.47	—	5.9	—	5.9	—
2.483	0.04	3.033	0.09			9.533	0.0845
2.683	0.09	3.50	0.15			10.017	0.140
2.917	0.15	3.54	0.18			10.05	0.164
3.282	0.25	11.18	0.23			11.45	0.197
4.50	0.40	12.03	0.226			12.07	0.20
12.084	0.405	12.33	0.234			12.36	0.203
12.367	0.405						
12.553	0.40						
12.70	0.39						
12.88	0.39						
13.033	0.395						
13.183	0.4						

against cH or cOH , curves are obtained by means of which the normality of acid or base corresponding to any hydrogen or hydroxyl ion concentration may be read

Titration of Glycine and of Sulfanilic Acid—An $N/10$ solution of

glycine was prepared, and 10 cc of this added to 15 cc each of the various solutions of HCl and NaOH. The resulting glycine concentration was thus 0.04. Table II gives the results with glycine. N

TABLE IV

0.04/0.043		0.02/0.02		0.10/0.04		0.04/0.08	
pH	n	pH	n	pH	n	pH	n
0.617	041	1.033	019	1.467	0841	1.525	0286
0.73	0395	1.18	0194	2.175	0532	1.825	0246
0.84	04	1.41	0170	2.433	0363	2.117	01724
0.99	039	1.62	015	2.625	0226	2.208	01174
1.125	039	1.97	014	2.73	01614	2.333	0092
1.31	0375	2.15	0108	2.833	011	2.39	0049
1.58	033	2.32	00715	2.88	0077	2.48	0027
1.91	0273	2.405	00506	2.97	0049	2.55	00017
2.21	0186	2.525	00301	3.017	00204		
2.36	0136	2.65	00076				
2.48	0087						
2.575	00634						
2.625	00363						
2.683	00092						
2.74	—	2.76	—	3.06	—	2.60	—
2.8	001	2.967	004				
2.85	003	3.217	009				
2.933	0075	3.583	015				
3.083	0133	8.867	025				
3.483	0266	10.38	0397				
4.11	039	12.067	041				
4.517	0412	12.367	041				
6.14	0433	12.55	039				
8.25	045	12.71	0397				
8.70	048	12.883	039				
9.175	054	13.03	0405				
9.45	060	13.183	040				
10.15	0748						
10.92	082						
12.05	0829						
12.32	0824						

is the original normality of the HCl or NaOH after dilution from 15 to 25 cc, and n is the number of mols neutralized by the glycine, per liter of mixture.

Table III gives, in condensed form, results obtained with certain concentrations of sulfanilic acid as well as certain other concentrations of glycine. The solubility of the sulfanilic acid prevented making an $N/10$ solution at room temperature, so the solution was made up at 40°C using such a volume that 0.1 mol would occupy a volume of 1 liter at 20°C . A quantity was pipetted from this solution at 40° such that it would occupy a volume of 10 cc at 20° . As may be expected, acid titration of sulfanilic acid has little meaning, since at the pH where any effect may be expected, a very small

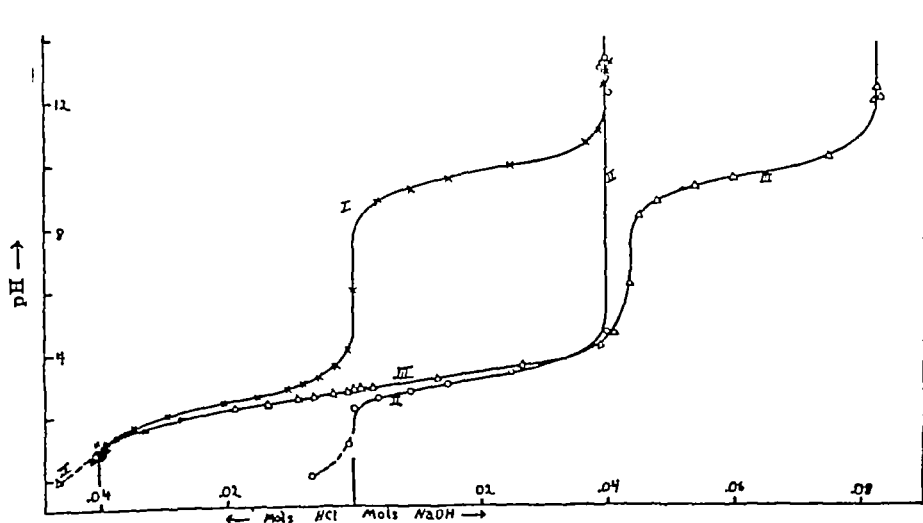


FIG 1

change in EMF corresponds to a large quantity of sulfanilic acid neutralized. Thus, except for glycine, only alkaline titrations are included.

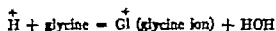
In Tables III and IV, n represents mols of HCl neutralized when it is above the center line, and mols of NaOH neutralized when below this line.

Table IV, which is analogous to Table III, gives results obtained from the titration of various mol ratios of glycine and sulfanilic acid. The concentration of glycine is given first in the ratios, *i.e.* the ratio 0.04/0.043 means a mixture containing 0.04 mol glycine and 0.043 mol sulfanilic acid per liter.

The curves in Fig 1 are plotted from data obtained by titration of the 0.04 N glycine (Curve I), the 0.04 N sulfanilic acid (Curve II), and the 0.04/0.043 mixture of glycine and sulfanilic acid (Curve III). The alkaline titration of the sulfanilic acid (Curve II) is plotted on an abscissæ scale 43/40 that of the other curves to compare its behavior easily with that of the mixture. The broken portions of the curves are drawn, not through directly determined experimental points, but through points calculated from the basic ionization constant of sulfanilic acid (6). Abscissæ give the mols of HCl, measured to the right of the zero line, or of NaOH, measured to the left of this line, neutralized, as calculated by the formula given above, while ordinates give the corresponding pH. Similar curves may be obtained by plotting in a like manner the data from the other titrations.

The striking thing about the curve is the high buffering action of the mixture of glycine and sulfanilic acid about a pH which, it will be shown, corresponds closely with the value that one would calculate for what has been termed the isoelectric point of the system, from the formula developed in the first paper of this series. Moreover, it will be noted that the behavior of the mixture as a seeming individual, at least with a characteristic curve differing from the curves of the components studied alone, is confined to that portion of the pH range between the respective isoelectric points of the components. At a pH above the isoelectric point of glycine the curve representing the behavior of the mixture is an exact duplicate of the glycine curve, displaced to the right, of course, by the amount of the sulfanilic acid concentration in the mixture. In the same way there is no reason to doubt that the curve below the isoelectric point of the sulfanilic acid, at a pH of 1.25 (6), and that of the pure sulfanilic acid would also duplicate each other, though unfortunately, with this substance, that portion of the curve cannot be easily experimentally realized.

Calculation of the Amount of Glycine Neutralized by Sulfanilic Acid
—The total amount of glycine neutralized can be estimated by considering the expression



where

$$\frac{(\overset{+}{\text{GI}})}{(\overset{+}{\text{H}})(\text{glycine})} = K$$

Reading the titration curve at various known ratios of glycine ion concentration to that of unneutralized glycine, K may be evaluated. For example, when the glycine is half neutralized, $K = 1/(\overset{+}{\text{H}}) = 223$, since 0.02 mol of glycine is seen to be neutralized at a pH of 2.35 or a cH of 0.0047. In a like manner for the following ratios the corresponding values of K are obtained

Ratios	pH	K
0.333	2.8	211
0.500	2.64	218
1.000	2.35	223
2.000	2.05	224
3.000	1.9	238
Mean		223

From the value of K the amount of glycine neutralized at any pH can be calculated. In the presence of NaOH this value is, through the pH range included in Table V, also the amount of glycine neutralized by the sulfanilic acid. When HCl is also present one must subtract from the total glycine neutralized the amount neutralized by the HCl. This can be satisfactorily approximated by putting it equal to the sum of the increase in glycine ion and the decrease in sulfonate ion at any pH, referred to their respective concentrations in the absence of the HCl.

Table V gives the results of such calculations for the mixture represented in Fig. 1. Above the center line the mixture contains NaOH, while below, it contains HCl. Glycine ion is represented by GI^+ , sulfonate ion by S^- , and their respective tabulated increments by ΔGI^+ and ΔS^- .

The mols of glycine neutralized by the sulfanilic acid are seen to pass through a maximum when there is neither HCl nor NaOH present. Such a mixture has a pH of 2.74. The theoretical value for

the isoelectric point of such a system, it will be remembered from the first paper of this series, is obtained from the expression

$$(\text{H})^+ = \sqrt{\frac{K (\text{HAOH})}{K'_2 (\text{HBOH})}} K_2$$

where, in the present case, K_2 is the acid ionization constant of the sulfanilic acid, 7×10^{-4} , and (HAOH) is its concentration in unionized

TABLE V

Concentration of HCl or NaOH	pH	\bar{G}	\bar{S}	$\Delta \bar{G}$	$\Delta \bar{S}$	Glycine neutralized by HCl	Glycine neutralized by sul- fanilic acid
0267	00033	00273	—	—	—	—	00273
0133	00083	0062	—	—	—	—	0062
0075	00117	00825	—	—	—	—	0083
0030	00141	00954	—	—	—	—	0095
00133	00158	01040	—	—	—	—	01040
0000	00183	01155	0119	—	—	—	01155
001	00186	0117	01175	00015	00015	00030	01140
002	00191	0119	0115	00035	0004	00075	01115
003	00207	0126	01085	00105	00105	0021	0105
006	00237	0138	0098	00225	0021	00435	00945
009	00266	0149	00895	00335	00295	0063	0086
012	00329	0169	00755	00535	00435	0097	0072
018	00439	0197	0059	00815	0060	01415	00555
025	0062	0232	00436	01165	00754	01919	0040
040	0124	0294	0023	01785	0096	02745	00195
060	0261	0341	0011	02255	0108	03335	00075

form, and K'_2 is the basic ionization constant of the glycine, 2.2×10^{-12} , and (HBOH) is its concentration in unionized form. Substituting the proper values, one is lead to the theoretical pH value for the isoelectric point of this mixture of 2.73 in place of the observed value of 2.74.

Other mol ratios yield analogous results. Table VI compares the observed pH of the isoelectric points of various mixtures of glycine and sulfanilic acid with the calculated values. These calculated theoretical values, it will be remembered, correspond to the pH at which the negative sulfonate ion concentration is equal to the posi-

tive glycine ion concentration They are obtained by solving the following two simultaneous equations for y

$$\frac{y}{c-x} \frac{x}{x} = K_a \quad \text{and} \quad \frac{K_w/y}{c'-x} \frac{x}{x} = K'_b$$

where y is the hydrogen ion concentration corresponding to the pH of the isoelectric point of the system,

x is the sulfonate (or glycine) ion concentration at this pH, which is the same in case of both ions,

c is the total sulfanilic acid concentration, and K_a its acid ionization constant,

c' is the total glycine concentration, and K'_b its basic ionization constant

TABLE VI

Concentration of sulfanilic acid.	Concentration of glycine	pH observed.	pH calculated
043	040	2 74	2 73
01	09	3 40	3 44
03	07	2 98	3 01
04	10	3 06	3 03
05	10	2 98	2 97
10	10	2 74	2 75
02	02	2 76	2 75
10	05	2 58	2 54
08	04	2 57	2 54
07	03	2 51	2 50

Table VII gives certain results of the same nature as those included in Table VI but for a few other pairs of substances In connection with the work presented in the following paper a sample of lysine was prepared from hydrolyzed casein The ionization constants of this substance are evidently not very accurately known, probably since it is difficult to prepare it with a high degree of purity Scudder (7) gives for K_a about 1×10^{-11} , and for K_b "less than 1×10^{-7} " From Tague's titration of lysine dihydrochloride (8) one may calculate the value of K_a , which is found to be 1.2×10^{-11} , but, though the second basic ionization constant can be obtained from his curve, the first, which is the significant one, cannot Solutions of the sample prepared for this work gave a pH to water of 8.8, which, using

1.2×10^{-11} for K_2 , gives the value 5×10^{-8} for the first K_1 . It is almost impossible to be sure that one has not a trace of sulfuric acid in such a preparation, however, and for the calculations of the theoretical pH values given in Table VII the value 7×10^{-8} was used. The agreement between the observed and calculated values of the isoelectric points of these systems, though still quite satisfactory, is not so good as in Table VI. This may be due partly to the problematical value of K_2 for lysine used in calculating the theoretical values, and partly to the fact that small changes in the mol ratio have a much larger effect on the pH than is true for glycine and sul-

TABLE VII.

		pH observed.	pH calculated.
0.04 sulfanilic	0.02 lysine	3.09	3.15
0.02 "	0.02 "	4.88	5.0
0.02 "	0.04 "	6.69	6.82
0.02 glycine	0.01 lysine	8.05	8.09

fanylic acid, and thus small errors in the total concentration, which may be the case for the lysine, will contribute largely to the discrepancies. The agreement is, however, considered good.

It will be noted that in the last instance the glycine is playing the rôle of the acid constituent of the mixture, as it is now at a pH above its isoelectric point.

SUMMARY

Electrometric titrations of glycine, sulfanilic acid, and various mixtures of the two have been made. These mixtures are shown to give a curve which, between their respective isoelectric points, is different from that of either substance. These mixtures have a maximum buffering power at a pH which can be theoretically calculated, and which has the characteristics of an "isoelectric point of the system."

Other pairs of ampholytes are shown to act in an analogous manner.

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PHYSIOLOGICAL ONTOGENY

A CHICKEN EMBRYOS

VII THE METABOLISM AS A FUNCTION OF AGE

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The rates of absorption, storage, and elimination of energy are perhaps the best indices that we possess of the vitality of a living organism. Since it has been frequently verified that $[A]$ absorbed energy = $[S]$ stored energy + $[E]$ eliminated energy, and since, by the chemical analyses of embryos at successive ages, the rate of storage has been ascertained (1), it remains only to obtain the catabolic activity of the embryo in terms of age. This has been done by the manometer method for the estimation of oxygen, and the present communication reports the results. The initial estimations of CO_2 production reported elsewhere (2) were admittedly subject to a number of unknown variables determinable with difficulty, such as variations in the concentration of CO_2 in the embryo and in the albumin and yolk, together with the contribution made by the carbonates dissolved from the shell. These values are functional to the carbon dioxide tension about the egg, and since, moreover, they cannot be estimated with great precision, a statistical analysis of the data from a very large number of eggs would be necessary. For these reasons we chose to measure metabolism by the oxygen consumption.

Method

A Warburg manometer was used, attached to a special glass vessel to contain the hen's egg. The egg rested upon glass tips projecting from the walls to suspend it above the bottom which was layered with 5 cc. of a 1.0 N NaOH solution.

The volume of each apparatus, and thus its constant, was obtained by the method of Warburg (3) Brodie's fluid was used in the manometer The volume occupied by the egg contents in the vessel, *i e* the whole egg minus the air sac, was assumed to be equal to the weight of the egg minus the weight of the shell (approximately 6 gm) In general, eggs of the same size and shape were selected

A control was made with each test, at first in the form of a fertile egg of 1 day incubation, but later, when this was found unnecessary, with a vessel empty except for the alkali The experiments were done in a constant temperature room, eliminating thereby the use of a water bath

A thermometer ground into each glass cover registered the temperature, which averaged approximately 39.0°C The small fluctuations which did occur were not found to affect appreciably the results Moreover, it was found that the large surface of alkali exposed provided for maximum absorption of CO₂ without the necessity of shaking Shaking for a minute prior to reading the manometer made no difference in the result Nor was it found, when thin rubber tubes in which cold water flowed were run along one side of the vessel to cool the wall over this area and thereby to initiate by convection a regular circulation, as in Barach's (4) recently constructed human oxygen chamber, that any acceleration of CO₂ absorption took place

About $\frac{1}{2}$ hour (the length of time being judged by the behavior of the control) was allowed for conditions to reach equilibrium After this time readings were taken at varying intervals during periods of 2 to 6 hours until repetition of approximately similar results made one confident of their reliability During the intervals between tests, the manometer and vessel were connected with an oxygen bag, so that the concentration of oxygen within the vessel remained always the same

To obtain values for the rate of oxygen absorption per gm of body weight the following figures are necessary, (1) the constant for the vessel (previously calculated), (2) the manometer readings, (3) the weight of the whole egg, and (4) the weight of the embryo

One phenomenon was observed which we have not been able to explain The embryo of an incubation age over 16 days, even when connected with the oxygen bag, did not survive in the apparatus over 12

hours Their metabolism after 3 to 6 hours gradually fell If the vessel was fully opened to the air for a few minutes the embryo would revive Apparently it had nothing to do with a lack of oxygen, accumulation of CO_2 , or changes in the humidity, neither was there an accumulation of ammonia.

TABLE I
Metabolism of Chicken Embryos as a Function of Age

1	2	3	4	5	6	7	8	9	10	11
Age	No. of observations.	By experiment. O_2 per day per gm. wet weight.	Standard deviation.	From curve. O_2 per day per gm. wet weight.	O_2 per day per gm. dry weight.	Solid oxidized per day per gm. dry weight.	Solid stored per day per gm. dry weight.	Absorption per day per gm. dry weight.	CO_2 per day per gm. wet weight.	Resp. quot.
Days		cc		cc.	cc.	gm.	gm.	gm.	cc.	
6	8	50.0	2.1	50.0	896	0.444	0.665	1.11	29.9	0.60
7	5	38.4	2.3	43.0	735	0.364	0.584	0.95	29.6	0.69
8	3	36.7	1.5	39.0	628	0.311	0.510	0.82	29.3	0.75
9	10	39.2	1.3	36.5	562	0.278	0.478	0.75	29.0	0.79
10	7	32.8	1.2	35.0	500	0.248	0.465	0.71	28.5	0.81
11	7	33.9	1.2	34.0	442	0.219	0.465	0.68	28.0	0.82
12	9	33.9	1.2	33.2	378	0.187	0.465	0.65	27.0	0.81
13	5	32.4	0.7	32.5	322	0.159	0.465	0.62	25.7	0.79
14	5	30.3	0.6	31.7	259	0.128	0.447	0.57	24.0	0.76
15	7	34.3	1.3	30.5	209	0.103	0.395	0.50	22.0	0.72
16	11	29.0	1.4	28.7	175	0.087	0.320	0.41	20.1	0.70
17	5	26.1	1.0	26.2	152	0.075	0.250	0.33	18.1	0.69
18	6	21.1	1.3	23.2	131	0.065	0.215	0.28	16.2	0.70
19	3	20.5	0.3	20.0	113	0.056			14.2	0.71

Column 6 = figures calculated by the aid of values for the percentage of solid substance previously determined (Murray (1))

Column 7 = values in Column 2 divided by 2019.3 (amount of oxygen absorbed when 1 gm. of fat is burned)

Column 8 = figures previously obtained (Murray (1) Table III)

Column 10 = figures read from smooth curve previously obtained (Murray (5))

RESULTS

The results of the oxygen determinations (Table I) may be seen (Fig. 1) to demonstrate a decrease in metabolic rate per gm. of body weight with age. This conclusion confirms that reached when the carbon dioxide was determined, except that in a more precise analysis

and comparison of the results it appears that the oxygen estimations show a sharp fall of metabolism during the first days of the period under observation, whereas the carbon dioxide figures do not (5) As has been mentioned in the introductory remarks, however, numerous complications arising from the variability of unknown factors cast doubt upon the value of CO_2 elimination as a measure of catabolic change

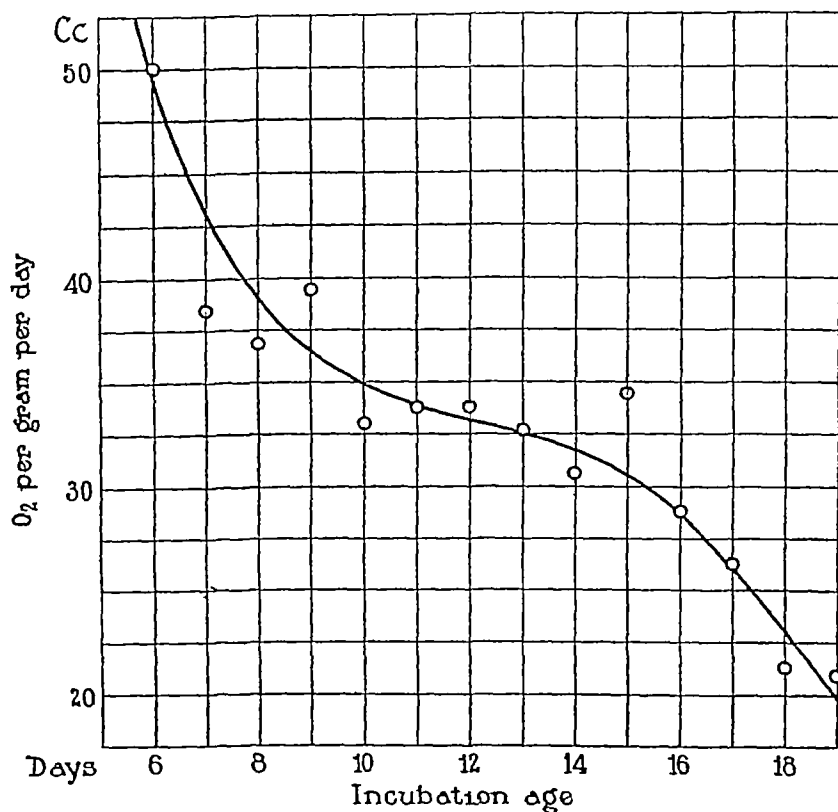


FIG 1 Oxygen consumption in cc per gm of wet weight of chick embryo per day, as a function of age

The oxygen determinations on the other hand were well controlled and presented no obvious factor to vitiate their use as indices of metabolic activity

To oxidize 1 gm of fat approximately 2000 cc of O_2 are absorbed, whereas to oxidize 1 gm of protein (966.3 cc) or starch (828.8 cc) about 900 cc of O_2 are used (6) The total oxygen consumption for

the first 19 days, estimated by graphical integration, comes to 2988 cc., which on the basis that only fat is burned during incubation leads to the conclusion that 1.48 gm. of dry substance (*i.e.* fat) is oxidized during that period. If only protein and starch were burned, it would require over 3.28 gm. to use the observed amount of oxygen. Previous chemical analyses have shown that approximately 1.62 gm. of substance is burned during the first 19 days, a figure which may now be accounted for on the assumption that 92 per cent of the metabolism is oxidation of fat, and the rest of protein and carbohydrate. This value is to be compared to 98 per cent fat oxidation found by measuring the CO_2 output. The former figure is probably more accurate.

During the last 5 days of incubation, when about four fifths of the total oxidation takes place, the respiratory quotient is approximately 0.71, which points to fat consumption, during this period. The earlier values for the respiratory quotient are somewhat higher (up to 0.81), but they are variable and it is uncertain whether they deserve consideration. The results point to some error during the first 3 days when the CO_2 figures, and thus the quotient, also seem to be definitely too low.

If we discard the carbon dioxide estimations in favor of these later O_2 determinations and assume as we may without undue error that catabolism is at the expense of fat, we arrive at some notion of the changes in the metabolic rate with age.

Regarding the organism energetically and dynamically, the amount of energy exchange measures its activity or vitality. Hence, the amount of energy stored plus the amount set free might be used as a criterion of aliveness. By adding the rate of storage in terms of weight (previously obtained) to the rate of elimination, likewise in terms of weight as measured by oxygen usage, one obtains the desired value, namely, the rate of dry mass absorption per gm. of body weight per day (Table I). It may be seen (Fig. 2) that there is a marked fall with age in the rate of absorption expressed in these terms. Reasons have been enumerated for believing that during the first half of incubation, when the amount of metabolism is small relative to the total metabolism during incubation but large relative to the weight of the embryo, there is a not inappreciable amount of protein and carbohydrate oxidized. If this were a fact a straight line rather than an S-shaped curve as graphically represented might be indicated.

The consumption of food during the early days is enormous. On the sixth day for instance the embryo absorbs over its own mass of dry substance. Assuming that the water content of the diet is ap-

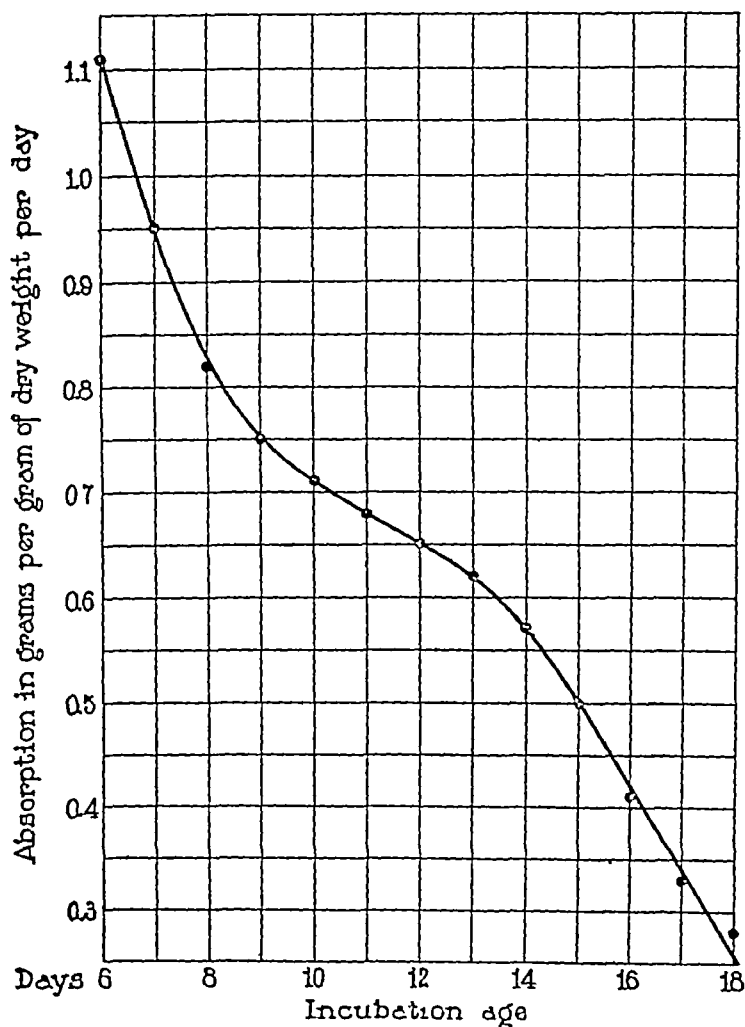


FIG 2 Absorption of solid matter in gm per gm of dry weight of chick embryo per day, as a function of age.

proximately that of the tissues, this would be equivalent to a mature man eating about 150 pounds of food per day. During the 12 days under observation, however, the percentage rate of absorption falls

to about 25 per cent (one-fourth its earlier value) According to Lotka, a mature meadowlark consumes about 6.6 per cent of its own weight a day (7) which would suggest a fall in absorption rate during the postembryonic period of a degree comparable to that which occurs during the 12 days before hatching

SUMMARY

1 The previous findings that the rate of metabolism per gm of body weight decreased with age, and that during the incubation period catabolism was mostly at the expense of fat, have been confirmed.

2 These determinations of the rate of oxygen uptake have afforded more precise values for the catabolic rate and thus permit estimations of the changes with age in the rate of absorption

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REGULATION OF THE HYDROGEN ION CONCENTRATION AND ITS RELATION TO METABOLISM AND RESPIRATION IN THE STARFISH

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The composition and condition of the body fluids of animals show departures from those of their environment usually corresponding to their degree of development. In the coelenterates and sponges a free circulation of sea water accomplishes the distribution of materials. The interior and exterior are then exposed to practically the same medium. In the starfish, however, there are a closed coelomic cavity and a digestive system which are not swept by a constantly renewed supply of sea water. The fluids contained in these spaces are similar to sea water, but modified by the enclosing tissue so as to be true components of the organism. The hydrogen ion concentration of the body fluids becomes progressively greater in the deeper regions of the organism, establishing a gradient from the interior toward the exterior. The normal reactions of these fluids are shown here to be optimal for several representative metabolic processes.

In the starfish there is a mouth-opening situated on the lower side, through which the soft cardiac portion of the stomach is extruded to surround food. In this stomach digestion proceeds until the food is sufficiently disintegrated to permit its withdrawal into the body. The partially digested food is then swept by ciliary currents through the pyloric caeca, where digestion and absorption are effected. Then, apparently the products of digestion pass through the wall of the caecum into the surrounding coelomic fluid. Here again ciliary currents, together with body movements serve for distribution (Irving 1924-25).

The investigations presented were carried out with two forms of

starfish common in the vicinity of the Hopkins Marine Station at Pacific Grove, California. These forms were *Pisaster ochraceus* and *Patiria miniata*. *Patiria* is short-armed, thin-walled, and much more tractable in aquaria. It was therefore used for most of the experiments, although many of them were also tried on *Pisaster* with corresponding results.

Normal pH of Stomach, Cæca, and Cælonic Fluid

The following preliminary experiments showed that the stomach is distinctly more acid than sea water. Mussel meat stained with neutral red takes the deep red color of a pH less than 7. During cardiac digestion of such meat, the red color is retained, and the stomach walls take and keep for several days a pink color indicating a hydrogen ion concentration near neutrality. Several times I was able to pipette enough fluid from the stomach through the mouth to show a pH between 7.3 and 7.5 with phenol red. This fluid is already much more acid than the sea water with which it is in such relatively close contact.

After ingestion of stained mussel meat the oral lumina of the cæca stain deep red with neutral red, the remaining regions being naturally too dark to show the dye. The red color remains conspicuous in the lumen for a week, during which time this particular cæcum region plainly remains more acid than sea water, but not far from neutrality. Although the neutral red color in the dark cæca is difficult to compare directly, the stained cæcum changes color distinctly when placed in a more alkaline or acid buffer solution. A series of cæca stained by ingestion of neutral red were placed in buffer solutions, and, from observation of the solution producing the least change, the cæcum pH was estimated to be between 6.6 and 7.0. This is quite contrary to the observations of Roaf (1909-10)¹ who declares that the cæca of *Asterias rubens* become alkaline after digestion is completed.

Samples of cælonic fluid, which surrounds the cæca in the body cavity, showed the following acidities:

<i>Patiria</i>	pH 8.0	7.7	7.6	7.6	7.6	7.6
<i>Pisaster</i>	pH 7.5	7.6	7.5	8.1	7.6	7.6
Sea water	pH 8.3					

¹ Roaf (1909-10), p. 448

These are typical of many determinations taken at various periods before and after feeding, and, considering the difficulties from mixture with sea water and a slight opalescence, indicate a consistent normal close to pH 7.6. McClendon (1916-17) found the pH of coelomic fluid of the sea urchin *Toxopneustes variegatus* between 7.7 and 7.8, while Crozier (1918) found the coelomic fluid of the holothurian

TABLE I

Changes in the Hydrogen Ion Concentration of Sea Water Caused by Excised Cæca

Experiment No.	0 hrs.		20 hrs. Cæca condition.		45 hrs. Cæca condition.	Comparative activity
	pH	pH		pH		
1	5.0	6.0	Dead	5.8		0
2	5.6	6.3	o.s. active i.s. dead.	5.8	o.s. slightly active. Digestion started.	1
3	6.0	6.35	o.s. active. i.s. active.	6.0	o.s. slightly active. i.s. dead.	2
4	6.2	6.35		6.2	o.s. active. i.s. dead.	3
5	6.4	6.4		6.2	o.s. active. i.s. slightly active.	4
6	6.6	6.35		6.2	o.s. active i.s. slightly active	3.5
7	6.8	6.55	o.s. active. i.s. active.	6.2	Dead	0
8	7.0	6.85		6.7	o.s. slightly active i.s. dead	1
9	7.4	6.95	o.s. active. i.s. active.	6.8	o.s. active. i.s. active.	4
10	7.8	6.9		6.7	o.s. active i.s. dead	1
11	8.4	6.9	o.s. active. i.s. active.	6.4	o.s. active i.s. dead.	1

o.s. = outside toward coelom i.s. = inside

Stichopus mæbis at 7.6. Coelomic fluid is also different from sea water in its salts. The freezing point depression of a sample from *Patiria* was determined by Dr J. P. Baumberger to be 1.885, compared with 2.075 for sea water.

These observations show that the fluids and organs of *Patiria* and *Pisaster* are maintained at an acidity quite different from the sea water.

The Optimum for Survival

After the discovery that the internal organs and fluids have each a normal, regular pH, they were next subjected to environments varying in this respect. In order to start under reproducible conditions, sea water acidified with HCl was aerated until a constant pH was reached, indicating the arrival of the solution at carbon dioxide equilibrium with the air.

TABLE II

Changes in the Hydrogen Ion Concentration of Sea Water Produced by Excised Cæca

Experiment No	Start	17 hrs	21 hrs	45 hrs
	pH	pH	pH	pH
1	5.0	6.2	6.1	6.0
2	5.4	6.3	6.3	6.1
3	5.8	6.3	6.3	6.1
4	6.0	6.7	6.7	6.2
5	6.2	6.3	6.3	6.1
6	6.4	6.3	6.3	6.2
7	6.6	6.3	6.3	6.1
8	6.8	6.7	6.7	6.7
9	7.0	6.8	6.7	
10	7.2	6.8	6.7	
11	7.4	6.8	6.7	6.6
12	7.6	6.8	6.8	6.6
13	7.8	6.8	6.8	
14	8.0	6.6	6.5	
15	8.2	6.7	6.7	
16	8.4	6.7	6.7	

To such solutions in test-tubes, excised cæca were added, one in each tube. Table I shows the changes which characteristically occurred in these experiments, and Table II extends the results. The condition of the tissues was determined in each case by examination with a binocular dissecting microscope.

These experiments show a tendency of the excised, living cæca to alter the medium toward a pH of about 6.7. When the pH fell below 6.7 to about 6.3, disintegration and injury to the cilia were apparent. The halt at pH 6.7 is too distinct to represent gradual

decomposition, and the subsequent rapid fall to pH about 6.3 indicates a change to a new status. Probably the lower pH, which is concomitant in appearance with physical signs of tissue death, represents the stage of autolysis. It is apparent that the living animal maintains a rather definite normal reaction of its caeca, and that the caeca themselves when excised are capable of producing the normal reaction in sea water media initially quite different.

All of these tests dealt with living tissue, except as indicated. It was not a matter of autolysis or putrefaction because of the persistence of ciliary action and the absence of the conspicuous odor of dead marine animals. These soft tissues disintegrate and produce unpleasant odors almost immediately after ciliary action ceases.

The Optimum pH for Digestion

Many of the older physiologists investigated the problem of digestion in echinoderms and produced conclusions of quite contradictory nature. Abderhalden (1911²) lists seven different enzymes named in echinoderms by various workers. The names appear, however, to be usually assigned by analogy with mammalian enzymes on the basis of substance digested and conditions of action, quite apart from normal conditions. Beyond these analogies, based often on rather indefinitely reported observations, there is little precise evidence of the nature of the enzymes. It is clear that the caeca produce digestive enzymes (Frédéricq, 1878, Cohnheim, 1901, van der Heyde, 1922, 1923). These enzymes easily bring about autolysis of the organs, but extracts behave in an extremely slow fashion. Furthermore, the technique applied to their study has not been of the sort calculated to give quantitative results.

Frédéricq (1878) first clearly showed that proteolytic digestion by the caeca occurred near the neutral reaction. Other invertebrates probably carry on digestion naturally near neutrality, and not in the strongly acid condition where pepsin finds its optimum. Darwin's observations on digestion in the earthworm show a normal reaction near neutrality (Darwin, 1890). Bodansky and Rose (1922³) extracted

² Abderhalden (1911), p. 538.

³ Bodansky and Rose (1922) p. 475.

a digesting substance from jelly fish which had one optimum for gelatin liquefaction at pH 2.6, and another, much more favorable, at pH 7.3. The optimum at pH 2.6 seems too far removed from the normal reaction of sea water, which circulates so freely through the animal, to be important under vital conditions. The empty stomach of *Stichopus* was found by Crozier (1918) to have a pH between 5.0 and 6.5, while during feeding it ranged from 4.8 to 5.5. Crozier remarks that this animal's calcareous diet may constitute a special condition which is met by the relatively strongly acid digestive juices. Roaf's observations of $\text{CH } 10^{-4}$ during digestion in *Asterias rubens*¹ suggest an acidity far greater than any which I observed in *Pisaster* or *Patiria*, although they are similar forms. Van der Heyde (1922) observed in the stomach of *Asterias* pH 7.1, 7.6, and 7.7, and in the cæca 7.3. The chemical methods and biological conditions involved in determining the normal hydrogen ion concentration during digestion must be rather precisely established, so that many casual observations require critical examination. From the available evidence and the biological conditions a natural reaction far from neutrality would be unexpected.

Because of the difficulties found in separating active extracts, digestions by excised surviving cæca *in vitro* were used. These are here nicely applicable because of the appropriateness of the cæca and the excellent criterion of their survival in ciliary activity.

Pipettes were prepared with their tips protected by rubber tubing, the opposite end bearing a short length of rubber tubing and a pinch-cock. 2 cc. of the solution to be digested were introduced into the pipette, its lower end stopped, and the pinch-cock closed. By slightly withdrawing the closed rubber tube from the pipette the solution could be retained without loss. Next the rubber-protected end of the pipette was inserted into the open (pyloric) end of a previously weighed cæcum and the cæcum tied on with silk. The cæcum was then drained and transferred to a test-tube containing 10 cc. of sea water prepared at constant pH. The pipette and cæcum were suspended in the solution by a cork, the pinch-cock opened, and the solution allowed to run into the cæcum. The cæcum became distended and remained distended, if uninjured, for 2 days or more. In this way a sack of living digestive tissue was produced, with a

capacity in the case of large caeca of over 3 cc. Products of protein digestion diffused through the caecum wall and were determined by the Van Slyke method for amino nitrogen.

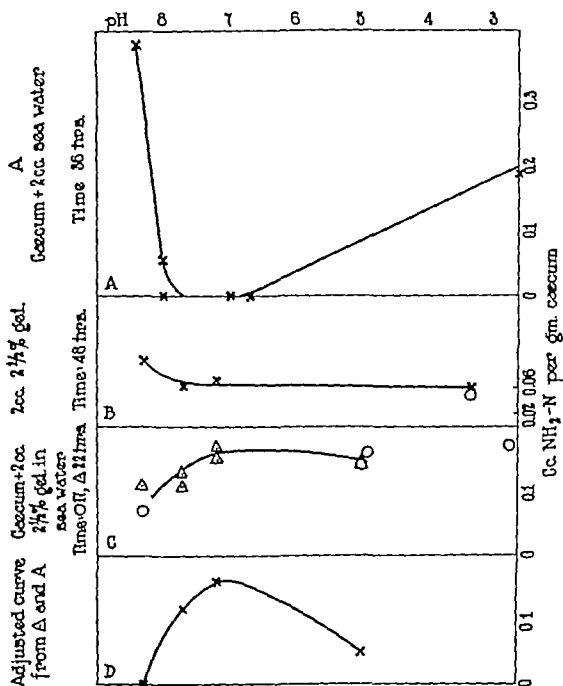


FIG 1

An appreciable production of amino nitrogen might be expected from the caeca themselves. In order to determine the normal amino

nitrogen production, 2 cc of sea water acidified and equilibrated by aeration were introduced through the pipette into the cæcum. The cæcum was then immersed in 10 cc of the same solution. After an interval of time a 2 cc sample of the external liquid was removed and its amino nitrogen determined. The new pH of the solution was also noted. In this way the amino nitrogen production of the cæcum alone could be determined. Data for the results are indicated in Fig 1, Curve A.

These control experiments indicate a minimum amino nitrogen production at about the neutral point, increasing on each side. The neutral reaction has already been shown to be near the normal for cæca, and consequently tissue death and self-digestion would be expected to be less. Bacterial action is not excluded, but if present is least effective in producing amino nitrogen at neutrality. Evidently also the energy consumed by the tissue during survival is not produced from protein, or the amino nitrogen would increase at the point of greatest activity.

To determine the effect on food digestion of various hydrogen ion concentrations, a 2½ per cent solution of gelatin in sea water was used. Control determinations of the amino nitrogen production of this stock solution during 48 hours are shown in Fig 1, Curve B. 2 cc of the solution were added to 10 cc of the sea water used in the experiments. The time of these controls is double that in the experiments on gelatin digestion, and yet the amino nitrogen production is very small.

The digestive effect of cæca on gelatin was tried in solutions at various hydrogen ion concentrations. All sea water used was acidified with hydrochloric acid and then aerated to constant pH. At the time of digestion determination, pH was again determined and the results were found in accord with previous experiments. Results are indicated by Curve C in Fig 1, where two typical experiments are recorded together. The curve presents directly the appearance of an optimum for digestion near pH 7. The greater amino nitrogen production in acid reactions is obviously, from Curve A, attributable to autolysis. When a curve is adjusted by deduction of amino nitrogen production as in Curve A from that in Curve C, the resulting curve, D, plainly shows an optimum near pH 7.

After these indications that the optimum digestion conditions were also those of the normal organism, attempts were made to prepare a digestive extract whose activity might be compared at different acidities. Various extracts were prepared by permitting *cæca* to autolyze with alcohol or toluol. Products of such procedure caused only slow digestion, and the results did not show that any appreciable separation of the enzyme had been effected. As another method, many *cæca* were macerated with sand, spread on glass plates, and dried under low pressure at 40 degrees. The dried mass

TABLE III

Changes in pH of Sea Water Produced by Cæca during Different Time Intervals

Experiment No.	Weight of <i>cæca</i> .	Start.	Period 1		Period 2		Period 3.		
	gm	pH	hrs	pH	hrs	pH	hrs	pH	
Aerated	1	3.64	8.2	3.08	7.8	9.40	7.7	3.16	7.8
	2	4.37	8.0	3.00	7.5	9.40	7.4	3.16	7.3
	3	3.53	7.8	2.92	7.4	9.40	7.3	3.16	7.3
	4	5.34	7.3	2.84	7.1	9.40	7.3	3.16	7.2
	5	4.53	5.0	2.80	6.8	9.40	6.8	3.16	6.8
	6	4.53	3.8	2.70	6.1	9.40	6.4	3.16	6.5
	7	6.33	8.1	2.92	7.9	3.25	7.5	20.0	6.8
Sealed.	8	6.58	7.9	2.75	7.7	3.25	7.3	20.0	6.7
	9	6.13	7.7	2.66	7.4	3.25	7.1	20.0	6.7
	10	6.41	7.3	2.56	7.1	3.25	6.8	20.0	6.6
	11	6.42	4.9	2.25	6.2	3.25	6.5	20.0	6.4
	12	6.25	3.8	2.16	4.8	3.25	5.2	20.0	5.6

was then ground, but could not be redissolved or uniformly suspended on account of the fat present. Using this material in temporary suspension, the neutral reaction was obviously most favorable for the liquefaction of gelatin.

The Optimum pH for CO₂ Production

Having shown that the optimum reaction for ciliary survival and digestion corresponds with the normal tissue reaction of about pH 6.7, it would be expected that this reaction would be most satisfactory for metabolism in general. Respiration, measured by CO₂ production, is the indicator used here for metabolic activity.

For the experiments uniform amounts of weighed cæca were placed in Pyrex flasks with sea water of known pH in equilibrium with the air. One series was aerated, the other sealed with rubber stoppers to retain CO₂ as produced by the cæca. Changes in the sea water of the aerated series should be then attributed to non-volatile substances or to a buffer effect of the tissues themselves, changes in the sealed series would include these and the change from CO₂. During the experiments none of the tissues putrified, and the times were all less than had been previously shown quite compatible with survival.

TABLE IV

Changes in pH of Sea Water Produced by Cæca during Different Time Intervals.

Experiment No	Weight of cæcum	Start.	Period 1		Period 2		Period 3		Period 4	
	gm	pH	hrs	pH	hrs	pH	hrs	pH	hrs	pH
Sealed	1 80	8.6	5.59	7.7	1.75	7.6	2.25	7.6	12.9	7.1
	1 46	8.5	5.40	8.0	1.75	7.8	2.40	7.6	12.9	7.1
	1 62	8.5	5.25	7.6	1.75	7.3	2.40	7.1	13.0	6.8
	1 34	8.2	5.08	7.1	1.59	6.8	2.40	6.7	13.0	6.7
	1 38	5.0	5.00	6.4	1.50	6.5	2.40	6.5	13.0	6.4
	1 28	4.8	4.75	5.0	1.50	5.8	2.40	6.1	13.0	6.2
Aerated	1 88	8.6	5.92	8.3	1.42	8.3	2.75	8.3	12.4	7.8
	1 82	8.5	5.75	8.3	1.42	8.3	2.75	8.3	12.6	8.2
	1 61	8.5	5.59	8.2	1.42	8.2	2.75	8.1	12.6	7.8
	1 36	8.2	5.25	7.7	1.42	7.7	2.75	7.3	12.6	7.2
	1 38	5.0	5.00	7.2	1.42	7.3	2.75	7.0	12.6	6.8
	1 37	4.8	4.84	6.3	1.42	6.3	2.75	6.2	12.6	6.4

The results of these experiments, recorded in Tables III and IV, show in both the sealed and aerated series the previously observed progressive acidification of the solutions above pH 6.7 toward this reaction. In the aerated series the change is relatively slight after the first period, an effect which probably originates principally in neutralization by tissue substance.

In preparing acidified sea water solutions the excess base, and consequently buffer effect, were changed by addition of strong acid. Therefore only those experiments are comparable which were carried out with the same solution. These are recorded in the tables in the

same horizontal line. In such solutions of similar excess base, hydrogen ion concentration change is directly proportional to carbon dioxide change in the range considered (McClendon, Gault, and Mul

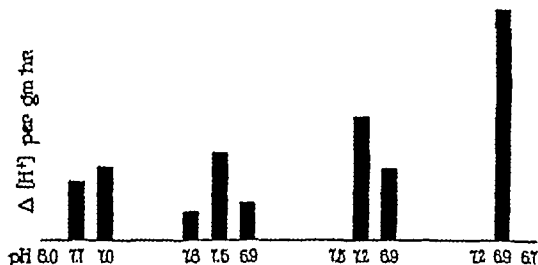


FIG. 2 (from Table III) $\Delta[H^+]$ per gm. per hour in sealed series, corrected by subtraction of $\Delta[H^+]$ of aerated series

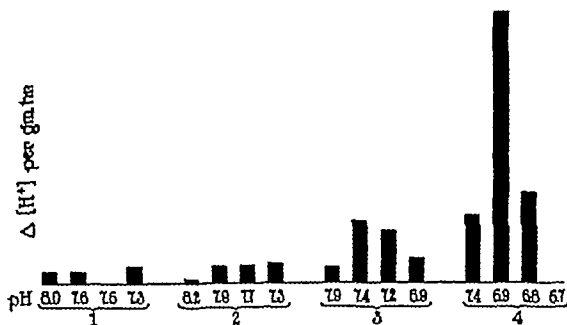


FIG. 3 (from Table IV) $\Delta[H^+]$ per gm. per hour in sealed series, corrected by subtraction of $\Delta[H^+]$ of aerated series.

holland, 1917) The progress of these changes in the sealed series is indicated in Figs 2 and 3 where vertical height represents change in hydrogen ion concentration per gm. of caecum substance per hour

These are corrected by subtraction of the change in the corresponding aerated experiment

The figures show in each experiment a small carbon dioxide production in the first period, followed by a much greater production in the second period, and usually by diminishing changes in subsequent periods. That respiration during the first period was so small is remarkable. It is apparent that the cæcum fits its medium better after some time for adjustment.

The reaction about pH 7, close above that found normal for the cæca, seems most favorable to carbon dioxide production. It is not possible, however, to judge this point, for the diminished excess base of the initially more strongly acidified series causes a correspondingly decreased buffer value.

The experiments show that carbon dioxide is the principal acid serving to bring the cæca to the normal reaction. The aerated series also changes slightly toward the normal reaction, but this change occurred almost entirely in the first period and may be attributed to the buffer effect of the cæcum substance. No evidence appears to suggest the production of an appreciable amount of non-volatile acid.

Conditions of acidity represented by pH less than 6.7 have already been shown to be unfavorable to the cæca. Where the reaction fell below pH 6.7 a sharp drop was noticed to a rather stable reaction about pH 6.3, which attended death and decomposition. In the aerated series at pH 5.0 reported in the tables the cæca were able to recover the normal reaction of pH 6.8, while in the corresponding sealed series recovery was impossible. Evidently here the first harmful attack is on respiratory activity, which is unable to eliminate carbon dioxide into acid solutions of such feeble buffer capacity without aeration. If the removal of carbon dioxide is facilitated by aeration, respiration is possible and the tissues can overcome the unfavorable conditions by neutralizing the acid.

For living starfish it was shown that the normal reaction in the cæca was about pH 6.7, for coelomic fluid which surrounds them, pH 7.6, and for sea water pH 8.3. To change sea water from pH 8.3-6.7 would require more than a fifty times increase in carbon dioxide tension (Henderson and Cohn, 1916). On the basis of this compari-

son the starfish evidently has quite a favorable pressure gradient for carbon dioxide elimination. The optimum might then follow only in consequence of the necessity of maintaining a carbon dioxide tension necessary to overcome the resistance to elimination. But each solution was initially in carbon dioxide equilibrium with the air, and all were altered by the *cæca* to or well on the way toward pH 6.7. In solutions of different buffer value this required carbon dioxide tensions of quite different magnitude so that the optimum was sought irrespective of the carbon dioxide tension it entailed. The optimum is therefore concluded to apply to processes of metabolism in general, and not to the mechanical elimination of carbon dioxide alone.

The optimum pH found is not, then, one merely favorable to respiration by the establishment of a large pressure gradient. It is an optimum, furthermore, which applies to metabolism in general. It is well known that tissues usually have optimal hydrogen ion concentrations for their most conspicuous reactions, but it did not necessarily follow that all of the metabolic processes should coincide in an optimum for the tissue as a whole, at which each individual process is most effective.

CONCLUSIONS

The *cæca* maintain for themselves a hydrogen ion concentration of pH 6.7, which is different from their immediate environment, the coelomic fluid. This in turn differs from sea water. The capacity for maintaining these differences approximately constant requires an efficient regulatory system. This does not infer the existence of a special organ or physical system in the organism, but a fact in the operation of its metabolism. Regulation of hydrogen ion concentration in living mammalian blood has proved to be an essential fact in the existence of the organism, although we cannot explain its entire operation or significance. A regulation similar in effect has been generally assumed to exist in all organisms, although it is seldom demonstrated. It is hard to conceive vital substances which could be effective and still incapable of the adjustment and preservation of their own reaction within a comparatively restricted range. It is probable that ability to regulate and maintain a constant hydrogen ion concentration agrees in general with the degree of development of the organism as a whole.

SUMMARY

The normal reaction of the coelomic fluid in *Patiria minata* and *Asterias ochraceus* is pH 7.6, and of the cæca, 6.7, compared with sea water at 8.3, all without salt error correction. A medium at pH 6.7–7.0 is optimum for the cæca for ciliary survival and digestion of protein, and is maintained by carbon dioxide production. The optimum pH found for carbon dioxide production is a true one for the effect of hydrogen ion concentration on the tissue. It does not represent an elimination gradient for carbon dioxide.

Because the normal excised cæca maintain a definite hydrogen ion concentration and change their internal environment toward that as an optimum during life, there exists a regulatory process which is an important vital function.

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Volume VIII of *The Journal of General Physiology* is a memorial to its founder Dr Jacques Loeb. This volume contains papers by Dr Loeb's pupils and his associates, together with a portrait and a sketch of his life. The volume is appearing simultaneously with Volumes IX and X. Number 1 of this volume will contain a biography of Dr Loeb. It is to appear after Number 6, and the page numbers will be roman numerals. The publication of this volume began September 18, 1925.

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THE JOURNAL OF GENERAL PHYSIOLOGY

AVENUE A AND 66TH STREET NEW YORK, N. Y.

THE EFFECT OF CERTAIN RESPIRATORY INHIBITORS ON THE RESPIRATION OF CHLORELLA.

By ROBERT EMERSON

(From the Kaiser William Institute for Biology, Berlin-Dahlem Germany)

(Accepted for publication December 2, 1926)

INTRODUCTORY REMARKS

This paper presents experiments on the effect of hydrocyanic acid, hydrogen sulfide, and carbon monoxide on the respiration of the green alga, *Chlorella*. The subject is of interest because all three of these substances are known to inhibit respiration specifically and reversibly in various organisms.

Hyman,¹ in a paper on the effects of potassium cyanide on *Planaria*, has reviewed the cases where direct measurements of the effects of cyanides on respiration have been made. Negelein² has published experiments on the effects of hydrogen sulfide, and Warburg³ on the effects of carbon monoxide.

It has been shown in this laboratory that these three inhibitors of respiration do not check the respiration of *Chlorella*. Warburg⁴ has shown this for hydrocyanic acid, Negelein⁵ for hydrogen sulfide, I have tried the effect of carbon monoxide.

As will be shown, this exceptional behavior of *Chlorella* vanishes when the alga is made heterotrophic.

Methods

The methods used in this work were essentially the same as those of Warburg and Negelein in their work on *Chlorella*. The alga was cultivated as described by them,⁶ in a water thermostat lighted continuously with three 75 watt metal

¹ Hyman, L. H., *Am J Physiol*, 1919, xlviii, 340

² Negelein E., *Biochem Z*, 1925, clxv, 203

³ Warburg O., *Biochem. Z* 1926, clxxvii, 471

⁴ Warburg O. *Biochem Z* 1919 c, 268

⁵ Warburg O., and Negelein, E. *Z physik Chem*, 1922, cli, 250

filament lamps, about 30 cm distant from the culture flasks. A slow stream of 5 per cent carbon dioxide in air was bubbled through the cultures. They were pure for the most part, but in control experiments with cells from cultures where no precautions were taken to exclude bacteria the same results were obtained as with cells from cultures known to be pure. The amount of *Chlorella* cells used so far exceeded that of any chance bacteria, that the latter did not affect the results.

Respiration was measured manometrically, in the dark. Measured quantities of cell suspension were pipetted into vessels of the type shown in Fig. 1. The vessels were connected by gas-tight joints with their respective manometers. The gas space above the cells, and the capillaries, as far as the manometric fluid, were filled with a mixture of 5 volumes per cent carbon dioxide in air before the

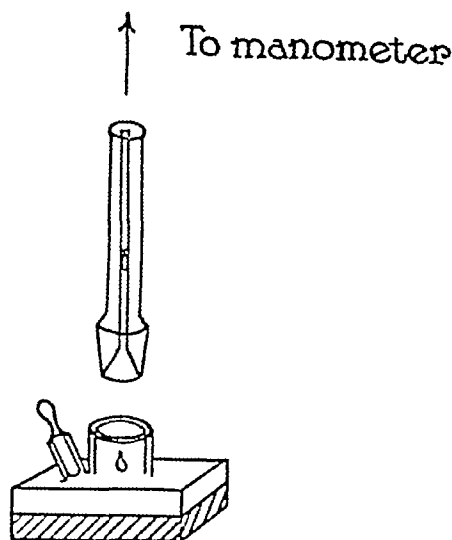


FIG. 1

system was closed. The vessels were shaken in a water thermostat at 20°C in order to maintain a state of equilibrium between the gas in the space above the cell suspension and that dissolved in the suspension. It was always fast enough so that an increase caused no change in the results. At intervals the shaking was interrupted for the purpose of reading the manometers.

The principle involved here is that oxygen is less soluble in the cell suspension than carbon dioxide. Thus, if the cells, in respiration, consume oxygen and give off the same amount of carbon dioxide, the pressure in the gas chamber will decrease, causing a change in the manometer (cf Warburg⁶ for a detailed discussion of this method).

⁶ Warburg, O. B. *Ann. Z.*, 1924, cliv, 51.

Experiments with Hydrocyanic Acid

The experiments with hydrocyanic acid will be described first and most completely. They are typical of those performed with hydrogen sulfide and carbon monoxide.

The concentration of hydrocyanic acid was 10^{-4} normal in practically all experiments. This is a convenient concentration for purposes of comparison, as Negelein has used it also. Its effect on

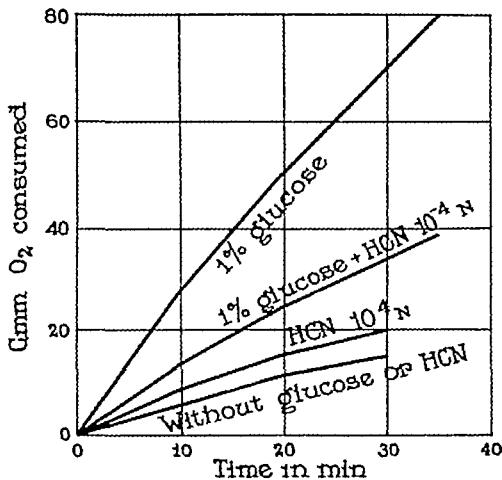


FIG. 2.

ordinary *Chlorella* cells (i.e. cells grown in an inorganic medium in bright light), is shown by the two lower curves of Fig. 2. Respiration is slightly accelerated. The two upper curves of Fig. 2 show the effect of the same concentration of hydrocyanic acid on the respiration of similar cells suspended in a solution containing 1 per cent glucose. It reduces their respiration over 50 per cent. Since all four curves represent the oxygen consumptions of the same amounts of cells, a

parison of the top and bottom curves shows that 1 per cent glucose about quadruples respiration

Osterhout⁷ and Krehan⁸ have shown that hydrocyanic acid has definite effects on the permeability of living cells. Hence it might be argued that the acid merely checks the penetration of the sugar into the cells, and not the oxidation process inside them. It may be shown in various ways that this is not the case.

Cells may be allowed to remain in a medium containing sugar for some time before the addition of hydrocyanic acid. When it is thus later added, the resulting inhibition is the same as when it is added with the sugar.

Another method is to grow *Chlorella* in a medium containing 1 per cent glucose. For the experiment, the cells are centrifuged off and transferred to an inorganic medium. The respiration of such cells is checked 40 to 50 per cent by 10^{-4} normal hydrocyanic acid.

The most conclusive method is to take autotrophic cells, grown in an inorganic medium, allow them to stand in 1 per cent glucose solution until the sugar has had time to penetrate (about 15 minutes), and then return them to the inorganic medium in order to measure the respiration and the effect of hydrocyanic acid. Respiration is reduced 60 to 70 per cent.

Organic substances of various sorts other than glucose have been tried, to see whether they would render *Chlorella* respiration sensitive to hydrocyanic acid. Those which, like glucose, call forth a respiration of three to four times the normal, were used successfully. Checking by hydrocyanic acid was the same as in glucose. Such substances are fructose, galactose, and mannose. All act almost exactly alike. The effect of the sugar on the respiration is very independent of the concentration. Glucose, for example, accelerates the respiration about four times, whether it is present in 4/100 per cent or 4 per cent solution. The inhibition by hydrocyanic acid is likewise independent of the sugar concentration.

Other substances tried gave slight acceleration of respiration (in general less than double). Many indifferent substances will cause this slight acceleration. But no real sensitivity to hydrocyanic acid

⁷ Osterhout, W. J. V. *Bull. Geol.*, 1917, LXIII, 77.

⁸ Krehan, M. *Internat. Z. für vergleich. Biol.*, 1914, 1, 189.

results Although respiration may be checked slightly at first, it is soon accelerated, just as with ordinary cells The substances tried were cane sugar, arabinose, dioxyacetone, glycoll, mannitol, and lactic acid Fig 3 shows the effect of hydrocyanic acid on cells suspended in a solution containing 1 per cent glycoll Glycoll was selected as typical of the indifferent substances. Various authors (e.g. Hyman¹) have stressed the reversibility of the hydrocyanic acid inhibition If the effect be irreversible, it is argued

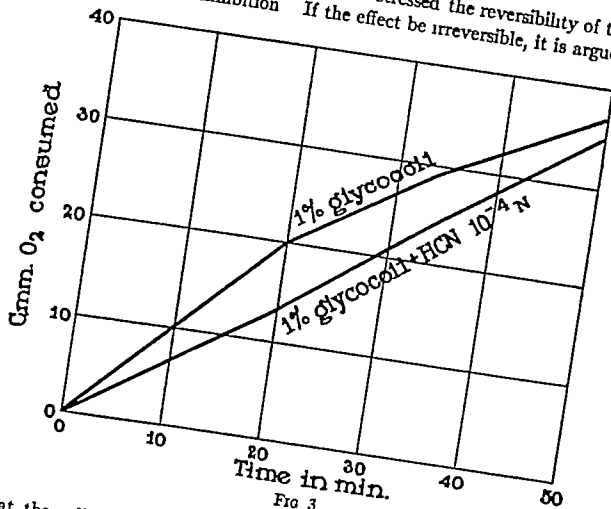


FIG 3

that the cells have been injured, and any inhibition of respiration cannot be regarded as specific. To test the reversibility in the case of sugar-containing *Chlorella* cells, the cells were subjected to the effect of hydrocyanic acid for 30 minutes, the checking of respiration measured, and then a stream of moist air was bubbled through the suspension, to remove the hydrocyanic acid. Respiration was then measured again, and found to be greater than in the control. The effect is therefore completely reversible.

Experiments with Hydrogen Sulfide.

Similar experiments were carried out with hydrogen sulfide and led to the same results as with hydrocyanic acid. Free hydrogen sulfide was formed in the cell suspension by the addition of an appropriate amount of N/10 solution of sodium sulfide. The solution

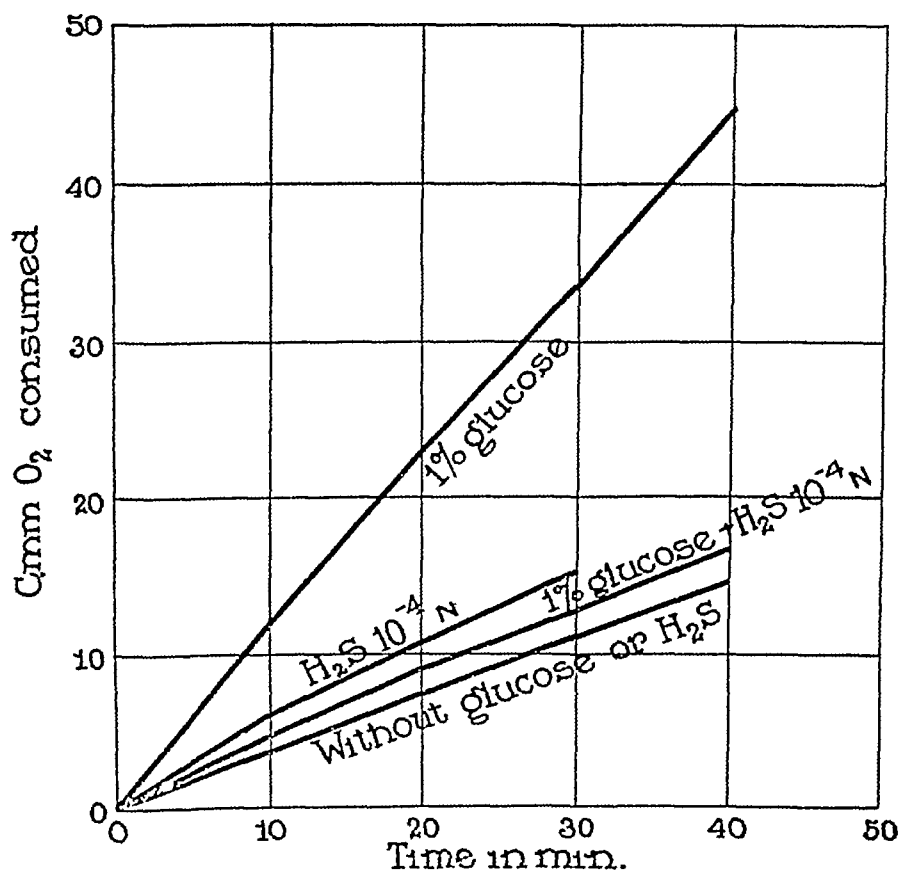


FIG 4

always 'contained' acid phosphate which reacts with Na₂S to form free H₂S. (For the calculation of the correct amount of sodium sulfide to add in order to achieve a given concentration of hydrogen sulfide in the solution, see Negelein's paper on the effects of hydrogen sulfide.) Equilibrium between solution and the gas chamber is established, a large amount of hydrogen sulfide remaining in the gas chamber

Fig 4 shows the effect of hydrogen sulfide on ordinary *Chlorella* cells and on cells suspended in 1 per cent glucose solution. It is essentially the same as Fig 1, which shows the corresponding curves for hydrocyanic acid.

The hydrogen sulfide effect was tested for reversibility, and it was found, like that of hydrocyanic acid, to be completely reversible. After removal of the hydrogen sulfide, respiration was greater than before it had been added.

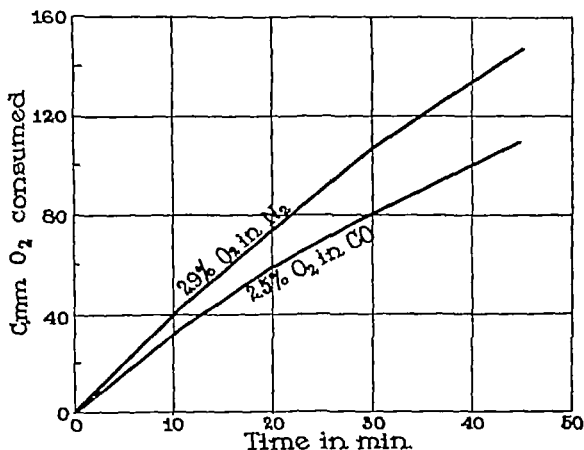


FIG 5

Experiments with Carbon Monoxide

In the experiments with carbon monoxide, a mixture of oxygen and carbon monoxide replaced the usual 5 per cent carbon dioxide in air used in the gas space. As control the same amount of oxygen in nitrogen was used. Fig 5 shows the effect of a mixture of approximately 2.5 volumes per cent oxygen in carbon monoxide on *Chlorella* suspended in a solution containing 1 per cent glucose. No curves are

given for the effect of carbon monoxide on ordinary *Chlorella* cells, for it does not affect their respiration

The inhibition of respiration by carbon monoxide is completely reversible. Carbon monoxide was removed by passing the oxygen-nitrogen mixture through the gas chamber

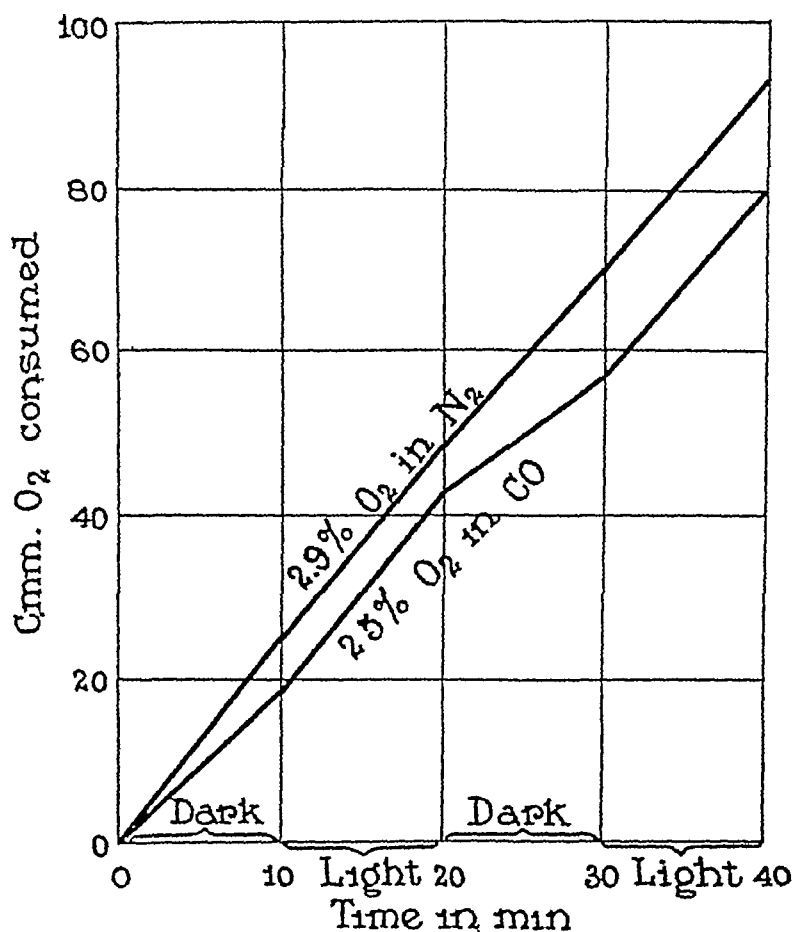


FIG 6

Warburg³ has shown that the inhibition of yeast respiration by carbon monoxide practically vanishes in light. This experiment cannot be performed with ordinary *Chlorella*, as photosynthesis takes place in the light. But yellow non-photosynthesizing *Chlorella*, practically chlorophyll-free, may be produced in a medium containing

1 per cent glucose and of low iron content. Such cells are completely heterotrophic, and their respiration is inhibited by hydrocyanic acid and hydrogen sulfide. Respiration is practically the same in light as in darkness. Fig. 6 shows the effect of successive periods of light and darkness on the respiration of such cells in 2.5 per cent oxygen in carbon monoxide, and in 2.9 per cent oxygen in nitrogen.

SUMMARY

Chlorella, when made heterotrophic by means of certain sugars, respire like other heterotrophic cells when subjected to the respiratory inhibitors, hydrocyanic acid, hydrogen sulfide, and carbon monoxide.

Whether the case of *Chlorella* is typical for green cells in general remains to be seen. Experiments with various other green organisms are being carried out, in hope of settling this point.

My thanks are due to Professor Otto Warburg for suggestion and criticism during this work.

THERMAL INCREMENTS FOR PULSATION FREQUENCY IN "ACCESSORY HEARTS" OF NOTONECTA.

By W J CROZIER AND T J B STIER.

(From the Laboratory of General Physiology, Harvard University Cambridge)

(Accepted for publication January 20 1927)

I.

For development of a theory of the temperature characteristics of vital processes it is necessary to deal with types of material in which it may be expected that experimental treatments can evoke one or another of a series of interrelated critical increments (Crozier, 1924-25, *a, b*, Crozier and Stier, 1924-25, *a*, 1925-26, *b*) In this paper we give an initial account of temperature relations in a kind of rhythmic activity which is in certain respects especially favorable for such tests.

Pulsatile organs, "accessory hearts," were first observed in the legs of juvenile aquatic hemiptera (*Notonecta*, *Ranatra*, *Corixa*, and others) by Behn (1835) These organs, located generally in the tibia just distal to the femoral articulation, or in the tarsus, were conceived by Behn to be a kind of "membranous valve" important in maintaining the peripheral circulation. Their "spontaneous" rhythmic movements were apparently confirmed, although misinterpreted, by Dufour (1835), they were discussed by Dugès (1838¹), and by Verloren (1847²), and a summary of the early observations on the "accessory hearts" in aquatic and other hemiptera was given by Edwards (1858) It was noted (Verloren, 1847) that in *Telligonia* the pulsatile activity might be interrupted for more or less lengthy periods, but that when active the frequency of the quick, sharp contractions exceeded that of the heart. The "hearts" were rediscovered by Mitchell (1858) Locy (1884) also described their movements, in *Ranatra*, *Notonecta*, and *Bdelostoma* subscribing to their rôle in connection with the circulation, he noted that the movements would continue for a time in amputated legs, and even when the "heart" was itself cut into parts. More recent

¹ Dugès 1838, p 441

² Verloren, 1847, p 82

observations, with figures, are given by Brôcher (1909). The existence of these organs is briefly referred to in entomological texts (Houlbert, 1920, Schroder, 1913), but nothing further appears to be known about these curiously inviting instances of perhaps myogenic rhythm. Among a number of possible sources of pulsatile organs in the bodies of insects (*cf.*, *e.g.*, Brôcher, 1916, 1917, 1919) they are by far the most amenable to experimental manipulation. They can be observed in the intact animal, and proper technique provides preparations in which the organs are active for days in detached legs. Six preparations may be gotten from a single individual, permitting for some purposes unusual control material.

In our experience the locomotor muscles of amputated legs of *Corixa* are likely to exhibit twitching movements, which interfere with the activities of the "accessory hearts," and in *Ranatra* the latter are difficult to see. Common species of *Notonecta*, however, are obtainable in large numbers, live very well in the laboratory, the "hearts" are easily seen, and the limb muscles of the detached legs are absolutely quiescent. The following account is based entirely upon the behavior of "accessory hearts" in the two swimming-legs of adult *Notonecta undulata*. It will be shown that although two preparations are obtainable from each individual these may nevertheless behave in quantitatively different ways, so that perfect control observations are not possible. But no necessity for such controls arose in the present work, which was designed to obtain primarily the temperature characteristics for pulsation-frequency during the survival of the isolated limb.

Our purpose was to discover if in different freshly isolated legs, and during the course of the irreversible death phenomena, there should not appear, as in the breathing movements of grasshoppers (Crozier and Stier, 1924-25, *a*), a series of differing critical thermal increments for pulsation-frequency, which might thus reveal certain of the inter-related processes involved in determining the rate of pulsation.

We were especially anxious to do this because of the possibility of obtaining information about typical governing processes in insect muscle, for comparison with central nervous activities (Crozier, 1924-25, *a*, Crozier and Fedeghi, 1924-25, *a, b*, Crozier and Stier, 1924-25, *a, b*, Fries, 1926-27). Although possibly containing nervous elements the "hearts" in isolated legs are obviously beyond central nervous influence. It turns out that the typical temperature char-

acteristics obtained for frequency of pulsation agree quantitatively with those known for respiratory phenomena (Crozier, 1924-25, *b*), and for breathing movements of insects (Crozier and Stier, 1924-25, *a*), but with the addition of a further member of this "set" of critical increments. They differ sharply from the value ($\mu = 12,300$) which is characteristic for frequency of heart contractions in arthropods (Crozier, 1924-25, *a*, Crozier and Fedenghi, 1924-25, *a*, Crozier and Stier, 1925-26, *b*, Fries, 1926-27). The associations observed between the four chief increments obtained are of additional interest for the analysis of vital processes from this standpoint.

II

The beating of the leg "hearts" is easily observed in the intact animal. The back-swimming habit of *Notonecta* makes it possible to prepare an individual by mounting it, ventral surface upward, upon a small block of hard rubber, to which it is attached by a small amount of vaseline, in such a way as to be at the surface of a vessel of water, with the legs outstretched in the surface film. Occasionally, movements of the appendages are seen, but the intervals between these more or less periodic movements are fairly long. The rate of pulsation in any one leg is very uniform, thus in one case, observed at frequent intervals over 24 hours, at 17°C, the time for ten contractions varied between 9.6 and 1.06 seconds, in another, between 5.2 and 5.6 seconds during 36 hours, frequently there is some decline in rate after 12 hours.

The beats are irregular for brief periods, sometimes a contraction is skipped. There is no evidence of diurnal rhythm. When the two swimming legs of one individual are compared, there is usually a distinct difference between them.

Animal No.	Leg	Time for ten pulsations, seconds									
N1 19	Left	5.4	5.6	5.3	5.5	5.5					
	Right	7.1	6.7	7.3	7.3	7.8	8.0				
N2 19*	Left	5.0	5.1	5.1	4.7	4.1	5.2	4.9	5.8	5.0	
	Right	4.4	4.9	4.7	5.6	5.1	5.5	5.7	4.4		
N3 17	Left	8.3	7.9	7.9	8.3						
	Right	7.4	7.0	7.6	7.0						

One leg-"heart" may abruptly cease operations for a time, the other continuing without pause. When the animal is stimulated, the beat may be hastened or retarded and irregularities appear.

The uncorrelated nature of the rates of contraction in the two legs and their independent variation speak for local rather than central nervous control of the activities of the leg-"hearts." Yet when the intact animal dies the frequency of the pulsations changes and to some extent their character also. When an animal in which the "heart" of one leg has been pulsating for some time at a steady frequency of 10.5 seconds for ten beats is completely covered by a thick layer of vaseline the rate remains constant for a time, but then, after several hours, the rate *suddenly* decreases to about one-half its former value, in both legs, and at this point the animal as a whole ceases to respond to stimulation. If left attached to the body the leg-"hearts" continue to beat for some 14 hours, but with decreasing although regular frequency. If detached from the body the legs show sustained pulsation for a much longer time. If the attached or isolated limb be swathed in vaseline no change in pulsation rate occurs.

These and other observations show that the rate of pulsation is locally determined, but that a secondary control, of nervous character, possibly, or dependent upon changes in the hemolymph pressure, is also important.

Two general types of result follow amputation of a swimming-leg. The rate of pulsation of the "heart" may remain the same as before amputation, the beat becoming at once more regular, and then, after about an hour (18°), the beat suddenly becomes much slower. In other cases the rate immediately after amputation may be about doubled, for several minutes, followed by sharp cessation of contraction which lasts about an hour, contractions are then resumed at about one-quarter of the original frequency. At the moment of pinching the leg with scissors the beats slow down, then become very rapid, then settle down to the initial rate. We have plugged the ends of amputated legs by exposing them to the air for 5 minutes, then by coating with vaseline, or the leg may be cut while imbedded in vaseline. No effects of these procedures were detectable.

It is important that the nature of the pulsatile movement of the leg-heart retains a very uniform character throughout the tempera-

ture range. Contraction is abrupt, relaxation slower, followed by an interval of quiescence. The contraction is maximal and always complete. The sheet of muscle fibers constituting the "heart" (cf Brôcher, 1909) is cap-shaped at the proximal end, from which a (?muscle) band passes to the femorotibial joint, distally, the "cap" spreads out into a flat contractile band. As a rule, contraction first appears at the apex of the "cap," and travels as a wave to the distal band. Occasionally the contraction wave is reversed. As death approaches the relaxation phase is prolonged, and the quiescent interval almost disappears, this also happens if the leg is sealed in a tube of water previously boiled.

III.

For observation, the isolated legs of *Nolonecta* were attached by vaseline to a glass slide placed in a small glass vessel of water. This vessel, having a flat bottom, was sunk in a chamber filled with water. The chamber was water tight, and addition of hot or cold water for regulation of temperature was by way of a coil of copper tube, with many small apertures, connected with an external supply. This chamber was immersed in a large stirred thermostat. Through the cover of the vessel containing the preparations the objective of the observing microscope passed, and also a thermometer reading to 0.01°. The objective was used as a water immersion lens, thus avoiding troublesome fogging, all the metal surfaces being coated with paraffin to obviate oligodynamic effects. The regulation of the temperature of the innermost vessel was secured by maintaining very slight differences between the temperature of the large thermostat and of the chamber immediately surrounding the observing vessel. Two, three, or four legs could be mounted together, and by adjustment of the microscope they could be viewed in succession or as desired. Light was reflected up through the thermostat and through a window in the inner box.

The muscles of an isolated leg, including the contractile fibers of its "heart," must of course be regarded as a system proceeding toward death, irreversibly. Therefore, if the relationship is to be obtained which exists between temperature and frequency of pulsation it is necessary to work rapidly in order to secure observations at a number

of temperatures before the underlying mechanism shall have changed materially. With these preparations it is possible to do so, largely because the latitude of variation in frequency of pulsations at constant temperature is so very slight.

After removal from the animal about 1 hour (at room temperature) is often necessary before the isolated "accessory heart" settles down to a steady rate of contraction. Not infrequently, however, this steady rate, lower than before amputation of the leg, is attained almost at once. A few successive readings of time for ten pulsations enable one to judge if the preparation is in a state suitable for experimentation. In order to discover the degree of constancy to be expected under uniform temperature, graphical records were obtained with the aid of a signal key and a chronoscope registering 10 second intervals upon a smoked drum. In this way a sufficiently precise record was obtained of continuous series of pulsations over periods of about $\frac{1}{2}$ hour. The high degree of constancy in "time for ten pulsations" in any one preparation is shown in the following table.

Temperature	Preparation	Mean time for ten contractions
°C		sec
16.4°	A ₁	19.0 ± 0.4
16.4°	B ₂	21.6 ± 0.2
16.4°	E ₂	19.2 ± 0.3
16.1°	I	17.95 ± 0.95
16.2°	K	15.7 ± 0.4

In this table the \pm limits give the *maximum* latitudes of variation, which average 4.9 per cent of the means. The same type of variation is evident in observations made at intervals, with a stop-watch, over periods of some hours. The maximum latitude of variation for any one heart is not over 10 per cent and is as a rule less than 5 per cent of the mean at a given temperature. This variation of course includes errors of observation. The same type of fluctuation is apparent throughout the temperature graphs. In good preparations constancy in pulsation rate is maintained for many hours, at uniform temperature.

IV

When the temperature is varied it is quite necessary to study each preparation individually. Averaging observations from different "accessory hearts" is not permissible. Even the two swimming legs from the same individual may differ significantly (Figs 7, 8), although they not infrequently give parallel results (Figs. 1 to 5).

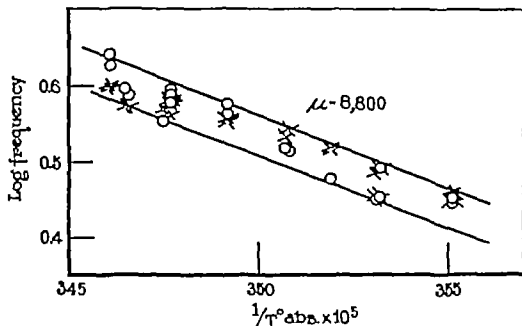


FIG 1 In this and succeeding figures the temperature characteristics for frequency of pulsation in the accessory leg "hearts" of *Notonecta* are obtained graphically by plotting *log frequency* (= $\log [100/\text{seconds for 10 beats}]$) against *reciprocal of absolute temperature*

Preparations Nos. 29 (circles) and 30 (crosses), (the latter having the rates multiplied by 2.0 for comparison) give $\mu = 8,800$. It may be noted that the latitude of variation with the large scale-units employed, corresponds to an extreme difference, at the left end of the graph of only 1.4 seconds in a mean of 23.4 seconds for ten pulsations.

In this way we have examined a large number of preparations, of which about 30 were studied in detail. The total number of observations was above 3,000. Since we desired especially to know the kinds of critical increments which might appear during the onset of death, it was necessary to avoid so far as possible the production of irreversible effects by exposures to very low or to very high temperatures. It was also necessary to work rapidly in order to make sure of time in

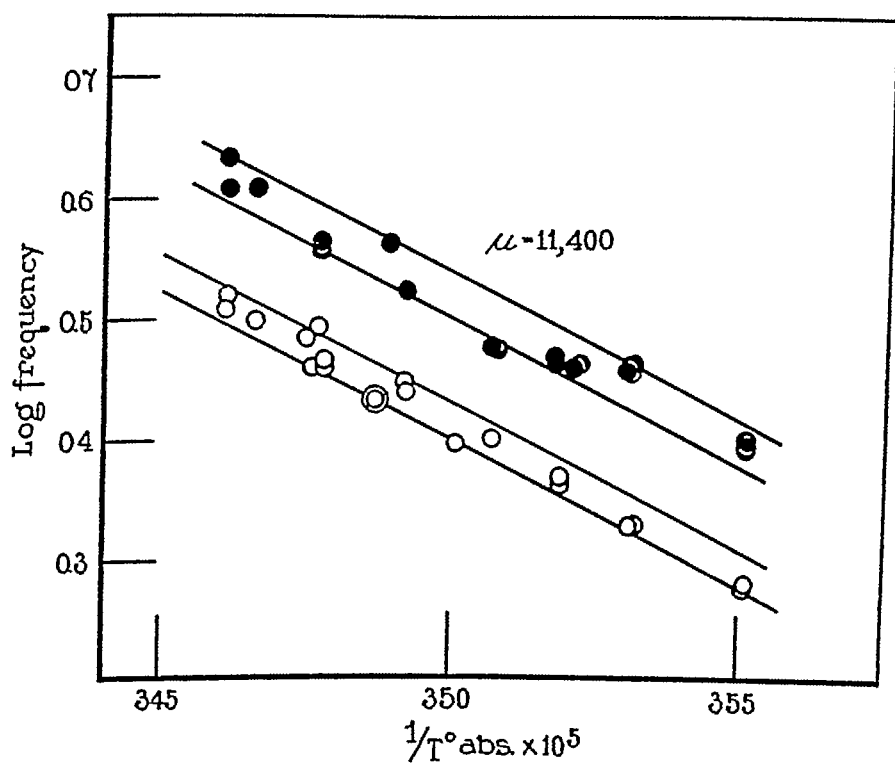


FIG 2 Legs Nos 27 (open circles) and 28, from the same individual, give $\mu = 11,400$

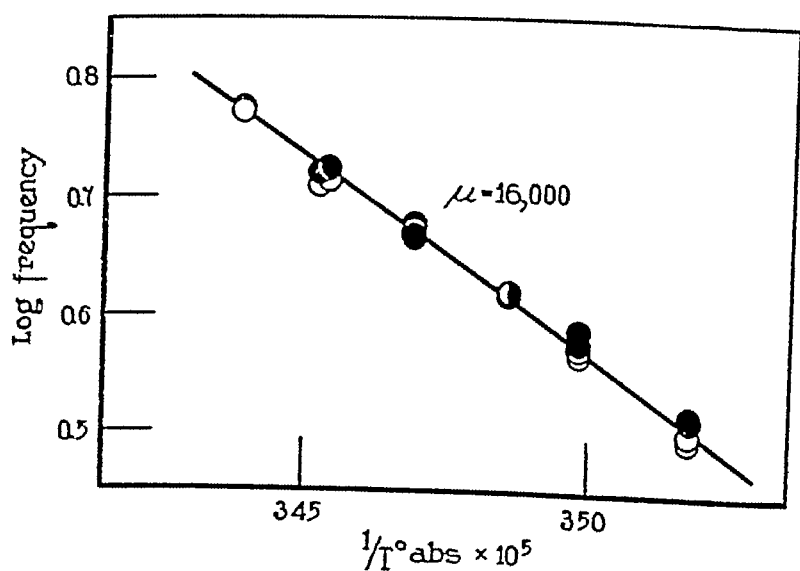


FIG 3 Legs No. 19 and 20 give sensibly identical results, $\mu = 16,000$

which to travel in reverse order the excursions up or down the temperature range before intrinsic change of temperature characteristic should supervene. The absence of great natural variation in rhythm, coupled with the non interference of spontaneous activities of the leg musculature, greatly facilitated such observations. The agreements in values of μ obtained show that confusion from these sources was successfully avoided.

The lower critical temperature for continued pulsation was 5° , in the isolated legs 20° was found a very definite upper limit for regu-

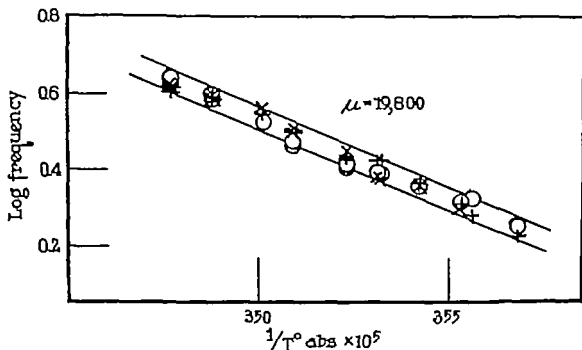


FIG 4 Three preparations, Nos. 50, 51, and 52 give $\mu = 19,800$ (The rates for No. 51 have been multiplied by 1.102 and those for No. 52 by 1.026) These were from different individuals

lar rhythm. In a number of preparations pulsations ceased at $9.5^\circ \pm 1.0^\circ$. The intermediate temperatures at which "breaks" appeared, indicating change of increment or of frequency, varied from 10.8° to 17.2° , the reason for this variation, as subsequently discussed, is found in the progressive exhaustion of the pulsating system, with induction of physicochemical changes independently of the thermal ones.

V

The results of these observations are sufficiently illustrated by the examples given in Figs. 1 to 11. Including those instances in which

"breaks" occurred, as exemplified in Figs 6, 8, and 11, the following is a summary of the critical increments found to describe the change in frequency of contraction as determined by temperature

Mean μ	Extreme range	Number of instances
8,190	7,900 to 9,150	14
11,350	11,290 to 11,500	4
16,200	15,100 to 17,000	16
19,800	19,600 to 20,000	4
24,530	23,000 to 25,000	5
32,200	30,000 to 34,000	12

The most precise determinations of μ are of course only obtainable from "runs" over a good range of temperatures, since in some cases such ranges were impossible to obtain in the present experiments, a certain variation in each value of μ as ascertained is to be expected. Certain details regarding the observations are discussed in the legends of the several figures.

The other magnitudes of μ , save 32,200, have already been recognized as occurring repeatedly in connection with a variety of biological processes (Crozier, 1924-25, *b*, 1925-26, *b*). Their reappearance here adds to the conviction that they correspond to chemical realities in living matter, perhaps to the heats of activation of commonly occurring catalysts (Crozier, 1924-25, *a*).

It will be noticed at once that the most frequently occurring magnitudes are, approximately, 8,000, 16,000, and 32,000 calories. Bliss (1925-26) has described a case in which temperature characteristics of these general magnitudes apply respectively to different parts of the total temperature range as affecting the time required for a particular developmental phase in *Drosophila*. This kind of progression tempts speculation. The additive nature of heats of activation is recalled. But we believe that for the present the fact may most safely be recorded devoid of speculative fringes. This is especially so because if the individual records are examined it is found that with any one preparation there is no orderly association or succession of values such as 8,000, 16,000, 32,000 in different parts of the temperature range, or at different times. The increment 16,000, for example,

may be associated with 32,000, or with 8,000, or 8,000 with 32,000. This speaks for a certain random association of the processes manifested by the appearance of the respective increments, with this restriction, however, the lower of two increments always pertains to the higher temperature range.

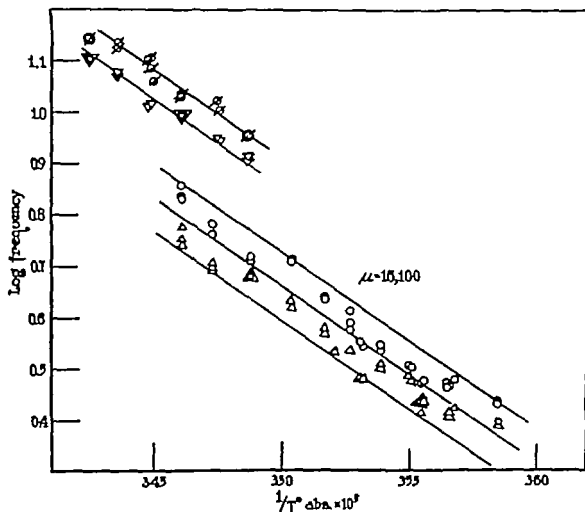


FIG 5 Leg-"hearts" Nos 31 and 32 from the same individual, give $\mu = 16,100$. The rate changed markedly after 24 hours, without noticeable change of μ , and by about the same amount in each leg.

In an isolated and irreversibly deteriorating system such as is presented by the isolated leg "heart" it is to be assumed that changes in μ should also be evident as a function of time.

By "a change which is a function of time" we mean to imply that the processes of exhaustion and death should influence the occurrence of "breaks" in the relation between pulsation frequency and tempera

ture The simplest illustration of such an effect is given by cases such as that shown in Figs 5 and 8 It is obvious that the differential effects of exhaustion upon each of several processes such as might possibly control the frequency of pulsation may result in these elements of the nexus governing pulsation being successively revealed as determining the relationship to temperature In numerous cases

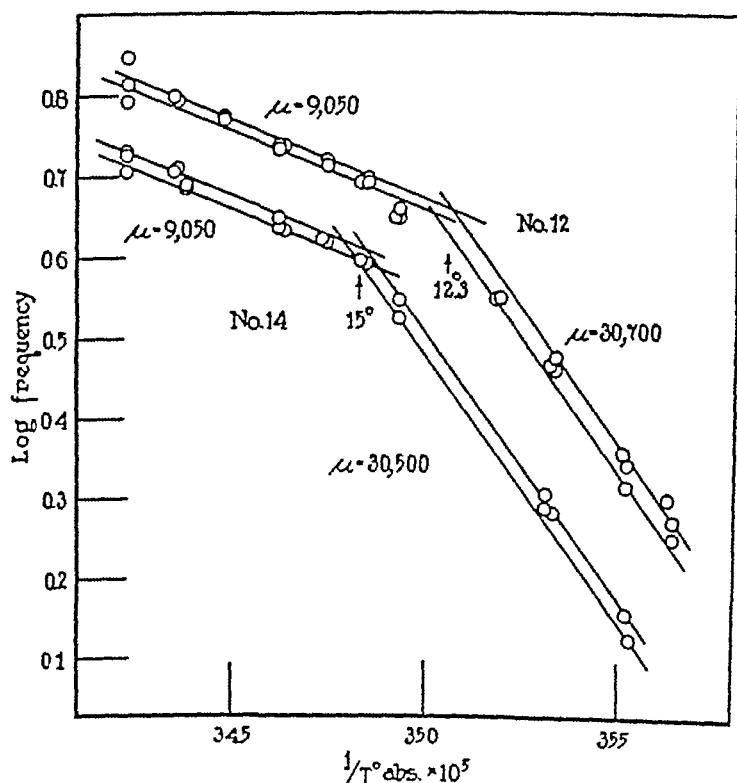


FIG 6 Two preparations from different individuals, show transitions from $\mu = 9,050$ to $\mu = 30,700$ at lower temperatures

where this sort of result is evident, reversibly, on passing from one zone of temperatures to another (Crozier, 1924-25, *b*, 1925-26, *b*) it has been assumed that it may be due to the fact that a catenary chain of reactions underlies the determination of frequency of pulsation (Crozier, 1924-25, *b*), the fact that the temperatures at which these shifts occur are not distributed at random (Crozier, 1925-26, *a*)

makes it necessary to suppose that physical changes, essentially of a grossly discontinuous character, are also implicated. In dealing with the *Notonecta* leg "hearts" it was expected that the degradation of the excised system would have similar effects, and that in consequence the occurrence of an intermediate critical temperature should be

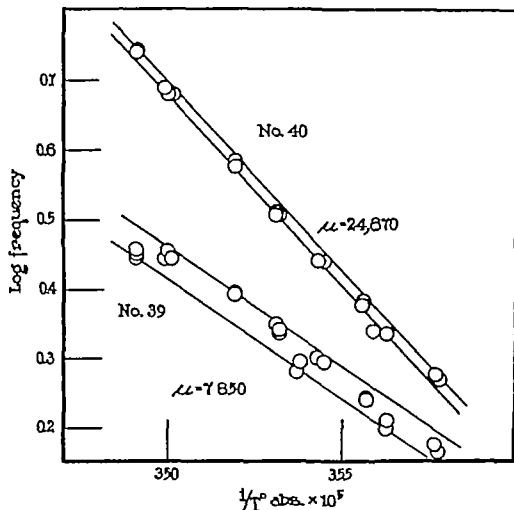


FIG. 7 Two leg 'hearts' (Nos. 39 and 40) from one individual give respectively $\mu = 7,900$ and $\mu = 24,900$. (The rates for one (No. 39) have been divided by a factor, 1.4 to permit unconfused plotting.)

blurred. This is the fact, but there is ample evidence, nevertheless, that in the neighborhood of 15° (10.8 to 17.2°) abrupt changes most frequently occur in the relationship between rate and temperature.

A further sort of complication in these curves is due to what we have characterized in other instances (Crozier and Stier, 1925-26, a) as a change of frequency or velocity without change of increment, or, what

is probably connected therewith in an intimate way (Crozier and Stier, 1925-26, *b*), a change in the latitude of variation at constant temperature without change of increment. This type of effect occurs in certain *Notonecta* preparations (Figs 9, 10, 11) and might easily confuse interpretation. To what extent these shifts may be due to change of pace-making location in the pulsating organ we cannot decide, in most instances, we feel, this explanation would not be correct.

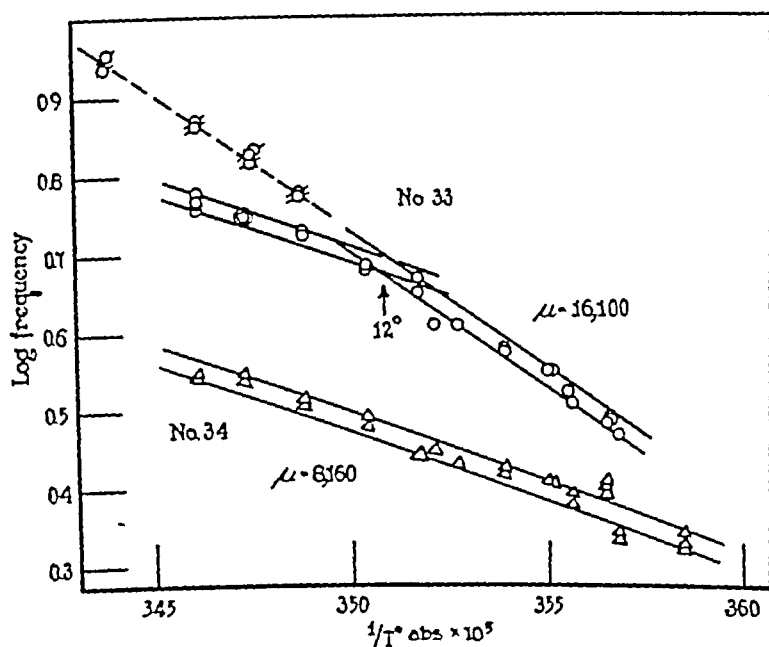


FIG 8 Two preparations from one individual give, in one case $\mu = 8,200$, in the other $\mu = 8,200$ above 12° , 16,100 below 12° , on reversing the course of the temperature changes (dashed line), next day, the increment 16,100 is now found to hold in this case above 12° . This type of change is one of those presumed to depend upon the irreversible progress of exhaustion in the isolated legs.

VI

The results of these experiments have been considered in terms of regularities in the nature of the influence of temperature on the frequency of pulsation in the leg-"hearts" and in a wide variety of other

objects. The magnitudes of the Arrhenius constant are not distributed at random. For this the explanation has been proposed that constants E or μ serve to characterize catalytic reactions governing the frequency of pulsation. It is possible to suggest a number of reasons why this sort of regularity "ought" not to be found. One method of dealing with the direct observations is to invent empirical formulae for their expression. One such has recently been proposed by Belehrádek (1926, *a, b*) in the form $y = a/x^b$, where x = temperature

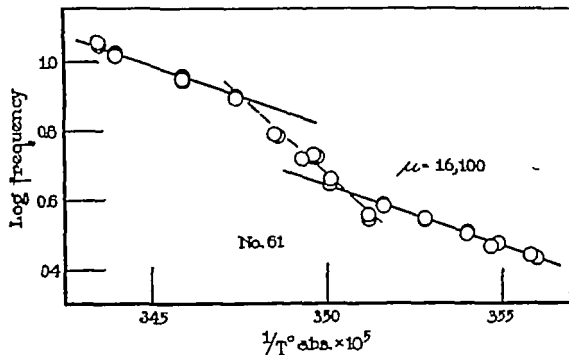


FIG. 9. A "break" in the curve of temperature relations was found in four cases, one of which is here shown which is similar to that earlier described by Crozier and Stier (1924-25, *b*) in connection with the breathing rhythm of Anurans. The course of the observations was exactly reversed on raising the temperature. (The slope of the fitted lines ($\mu = 16,100$) is the average of those fitting the upper and the lower segments.)

(Centigrade) and y = time necessary for a given phenomenon, and a and b are constants. This is obviously the well known empirical formula of Esson (Harcourt and Esson, 1895, Harcourt, 1912) for chemical reactions, with the substitution of the Centigrade temperature for $T^{\circ} \text{ abs.}$, it is difficult to conceive cogent reason, theoretically, for the proposed change, which simply requires making one of the constants larger without at all improving the fit. In addition to the

fact that this particular formula is of a type which can be made to describe *almost* any sort of curve not possessing too abrupt discontinuities, and that at best it has simply the status of an interpolation formula, there is the insuperable objection that it does *not* fit the facts. To illustrate this we may choose examples in which a large number of observations are available (Fig 12). The statement (Bělehrádek, 1926, a) that a single simple curve can describe instances of the sort

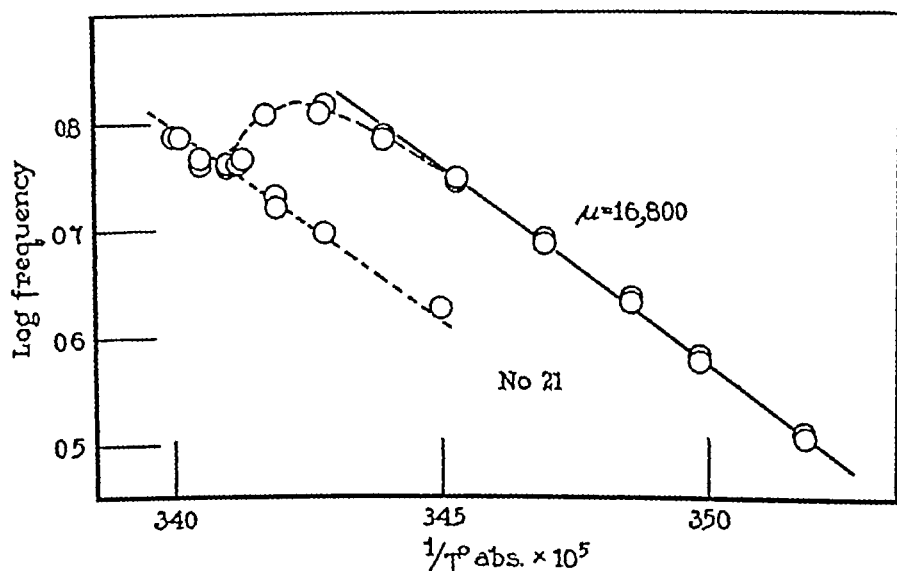


FIG 10 In this case the temperature was first raised to a point a little above 20° , there was thus induced a permanent increase in pulsation rate, without apparent change of increment. Precisely this effect was obtained in other instances. The temperature was changed at intervals of about 15 minutes, between readings, and in the region of changing rate the points consequently fall on a curve. It is to be understood that the whole extent of vertical shift would have occurred at constant temperature.

shown in Fig 6 is obviously futile (*cf* Brown, 1926-27). The attempt to employ his formula has moreover led Bělehrádek (1926, b) into the assertion that his constant b reflects the primary importance of viscosity in connection with the velocities of vital processes, and that it changes systematically with the age of the organism. The notion that the temperature coefficient of a given activity decreases or increases regularly with the age of the organism is simply untrue, as our

own experience with a considerable number of cases enables us to state quite definitely. We make no apology for choosing to rely upon the one type of equation which actually describes the data and which seems to stand a good chance of physical interpretation, and until some other comprehensive explanation is provided for the regularities which its use discloses we are not impressed by objections grounded, largely, on the argument that protoplasm is "too complex."³ The efficient answer to such very general objections is, that the complexity, or rather haziness, frequently resides in the mind of the observer, and can be dissipated by increased refinement of experimental procedures.

A more interesting sort of obstacle has recently been discussed by Murray (1925-26). In his experiments the temperature characteristics deduced for frequency of pulsation in cultured explants of chick myocardium failed to show uniformity, and, in the number of preparations studied, failed to be grouped about modal values. We are not familiar with the performance of such cultures, but it can be suggested

³ It is perhaps of interest, since the present material enables several points of some moment to be illustrated, to comment upon an objection sometimes voiced to the practice of fitting two or even three lines to segments of such data as are plotted, for example, in Fig. 6. It may be said that the implied transitions are too sharp. If one were really dealing with the effect of temperature upon a catenary series of reactions there should be a region of curvature connecting the two sensibly rectilinear zones. The answer is that in suitable instances just this indication of curvature is actually found. Most series of measurements exhibit a latitude of variation which makes it difficult to decide the precise nature of the union between the two straight lines. A number of series in the present experiments (e.g. Fig. 6) do however show this sort of "rounding off" when the latitude of variation is quite small. Objections to considering the whole sweep of such series as a single curve have been mentioned in a preceding paper (Crozier and Stier 1926-27, a). Sharp transitions of the sort implied in these broken graphs are of course well known in physics, we may refer to transition points and to curves of magnetic susceptibility. Moreover, as illustrated in a recent paper from this laboratory (Brown 1926-27) the data so fitted cannot be described by a single smooth curve when plotted directly as *velocities* against *temperature*. Again, as has been insisted previously (Crozier 1925-26 a) there is indication of "physical alterations occurring at just those temperatures most frequently found to be transition points on the thermal scale. The reason for the occurrence of just these critical temperatures must be sought in the properties of protoplasm as a physicochemical system.

that in such a preparation, essentially an unorganized mass structurally though exhibiting regularity as to pulsation, there must exist a number of possible pace makers. It is to be expected that the net result of their fluctuating dominance might obscure the preponderating in-

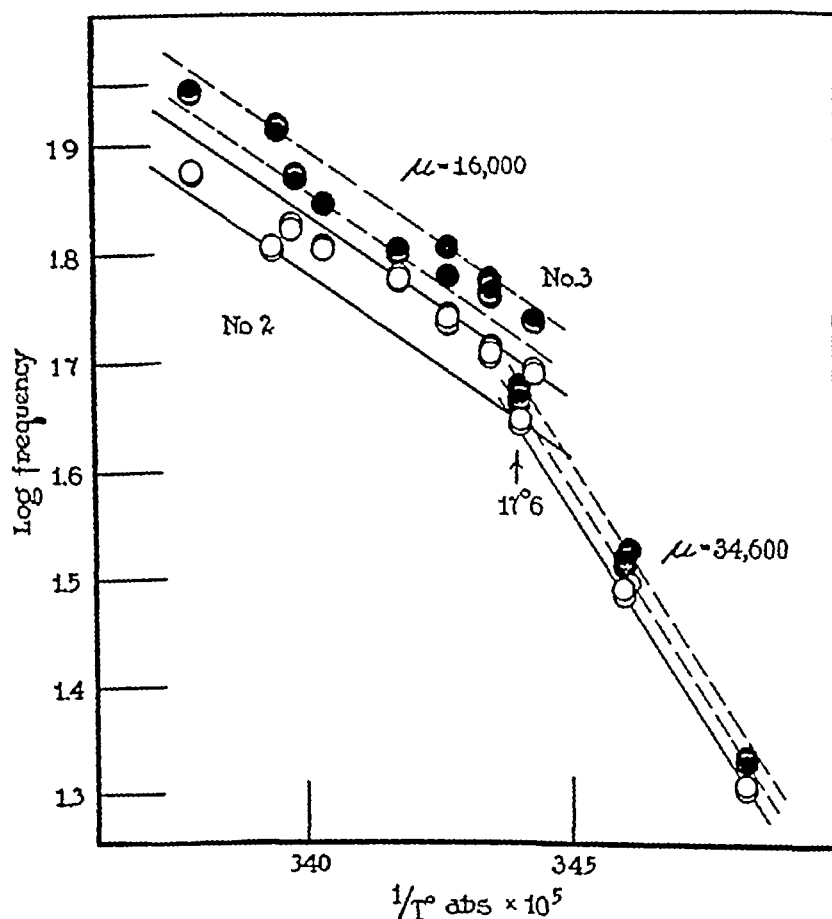


FIG. 11 Occasionally, instances were found in which an abrupt change of increment was associated with an abrupt increase of rate

fluence of any one, since it is fair to assume that their several inner metabolic states might be differently adjusted. As we have pointed out in detail in a following paper, this interpretation permits certain deductions which the figures in Murray's paper seem to us to justify

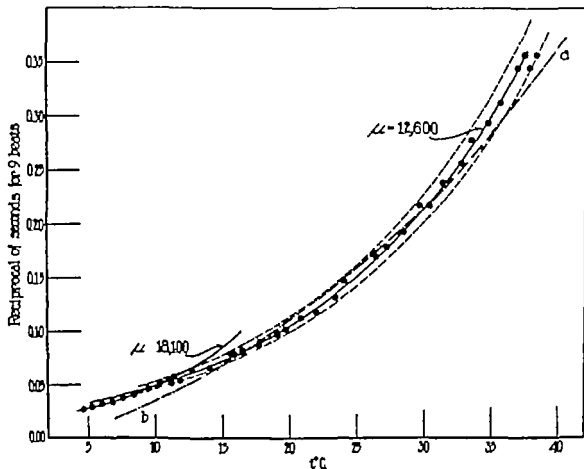


FIG 12 Data from one experiment (in Fries, 1926-27) on the frequency of cardiac contractions in *Blatta* are fitted by the Arrhenius formula with $\mu = 12\,600$ between 10°C , and 38°C . In the original presentation (Fries 1926-27 Fig 2) these data appear plotted as $\log \text{ frequency vs } 1/T^{\circ} \text{ abs}$, where the nature of the 'break' at 10 is more clearly apparent below 10° , $\mu = 18\,100$. The dashed lines are transposed from the lines defining the extreme latitude of variation in the log plot (Fries, 1926-27, Fig 2). The superiority of the logarithmic representation (vs $1/T^{\circ} \text{ abs}$) consists not only in the clearer appearance of conditions on either side of a critical temperature (e.g. 10 in this case) but also in the fact that the departures from the line of best fit are confined within a band the upper and lower edges of which are parallel to the central line (cf Crozier and Federighi 1925). The latter fact means that it is necessary to deal, in fitting the Arrhenius equation to such data, not with the absolute departures of the observed average rates or frequencies, but with the relative or proportionate divergences, this is of great importance if it be suggested that curves such as that in the present figure should be fitted by the method of least squares (cf, also Crozier and Federighi 1924-25 b 1925).

To curves obtained by the method just described there has been added (a, b, — — — —) the result of an attempt to fit the observations by means of the formula proposed by Bełehrádek (1926 a). This formula $\text{time} = A/(\theta^{\circ}\text{C})^B$ (or $\text{rate} = (\theta^{\circ})^B/A$) tested by appropriate plotting of the observations as $\log \text{ rate vs } \log (\theta^{\circ}\text{C})$. In such a plot the best fitting straight line was adjusted, and has then been transferred to the present figure. It is obvious that the fit is anything but significant nor can the adjustment of the ends of the curve be made such as to improve the fit from the nature of Bełehrádek's formula, correction of the lower end of the curve for example, merely increases the deviation at the upper end.

Extensive data upon the myogenic heart of *Limulus* larvæ (Crozier and Stier, 1926-27, *b*) have convinced us that the relation of pulsation frequency to temperature is of a perfectly regular sort, with definite and recurrent values of μ

VII

This paper is by intention an introductory account of material which it is designed to employ for certain types of experiment. But we may point to several conclusions which the preliminary examinations seem to justify. The rhythmic neuromuscular activities of arthropods, so far as tested, fall into two general categories as regards the critical thermal increments which they reveal. In one class are found movements of heart and of locomotor appendages and the rhythms of stridulation and of luminous display (Crozier, 1924-25, *a*, Crozier and Federighi, 1924-25, *a, b*, Crozier and Stier, 1925-26, *a*, Fries, 1926-27, and some other instances as yet unpublished). These we have reason to regard as controlled by nerve centers, and they show a high degree of consistency in providing $\mu = 12,200$ to $12,500$. Occasionally this value is associated with a higher one over the lower portion of the temperature range, $\mu = 18,200$ or $23,500$. On the other hand, movements of respiration (Crozier and Stier, 1924-25, *a*, and other data unpublished), presumably also determined by nerve center activity, but demonstrably of a different kind, consistently yield other increments,—those, namely, associated with cell respiration itself (Crozier, 1924-25, *b*).

The isolated leg of *Notonecta* with its "heart" is remote from central nervous control of the type pretty certainly involved in the movements of the dorsal vessel (*cf.* Alexandrowicz, 1926). We are not yet able to say just what the thermal relations are in the intact animal, but in the isolated leg-"heart" the increment $12,300 \pm$ does not appear among the several values encountered. On the other hand, the values actually found (Table I) are those characteristic of oxygen utilization, heat production, and CO_2 production. We do not conclude from this, of course, that the leg-"hearts" are therefore concerned with respiration, but merely that the metabolic activities underlying and controlling the rates of pulsation are certainly different from that determining the increment $12,300$.

It is notable that in material of this sort we should rather expect serious disturbance of rectilinear relationships between *log rate* and $1/T$, such that plainly curvilinear graphs would be gotten, or progressive shifts of μ . But the fact is that here, as in the case of grasshopper respiratory motions (Crozier and Stier, 1924-25, *a*) and in that of the heart of *Limax* (Crozier and Stier, 1925-26, *b*), when change of μ occurs it takes place abruptly and by a definite amount. This speaks strongly for the individualized character of the several processes which may control the rate of pulsation.

The presence of increments often associated with respiratory phenomena led us to attempt to alter the increment by controlling the oxygen supply. We were unable to obtain consistent differences by comparing hearts in legs immersed in water through which O_2 constantly bubbled, legs swathed in vaseline, or legs sealed in tubes with a small volume of water. The differences found are for the greater part attributable rather to the metabolic condition of the whole insect. This we expected to find influenced by laboratory confinement. It is of interest, therefore, to note that the increments $32,000 \pm$ were chiefly, although by no means exclusively, obtained from individuals not more than 2 weeks after collection. The increments 19,800 and 23,800 appeared only in the cases of legs from *Nolonecta* kept for some 2 months or more in the laboratory.

VIII.

SUMMARY

The frequencies of pulsation of the "accessory hearts" in the isolated swimming legs of *Nolonecta* were studied in relation to temperature, with the idea that in such organs central nervous control is impossible, and that in an isolated system irreversibly proceeding toward death it might be expected that further evidence would be found regarding the supposed specific significance of critical thermal increments. A number of values of μ are found commonly 8,200, 16,200, or 32,200, less frequently 11,400, 19,800, and 24,500. These values are definitely contrasted with that (12,300) typical for heart beat frequencies in arthropods. They exhibit interrelationships of the sorts already found in other cases. There occur also sharp irreversible changes in

frequency of pulsation, which may or may not be accompanied by change of increment. The net result is held to be confirmatory of the interpretation of thermal relations proposed in earlier papers.

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TEMPERATURE AND FREQUENCY OF CARDIAC CONTRACTIONS IN EMBRYOS OF LIMULUS

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I.

The heart of *Limulus* is classic as example of neurogenic cardiac rhythm (Carlson, 1905-06, 1909) The frequency of its pulsations may be controlled by influences affecting the heart ganglion alone When the temperature of the ganglion alone is varied (Garrey, 1920-21, a, b, c, 1921-22) the frequency of contractions adheres to the Arrhenius formula, μ in the equation

$$\text{Frequency} = K e^{-\frac{u}{RT}} + C$$

being $\approx 12,200$ (Crozier, 1924-25, a), in some preparations there occurs a "break" at 15° , with μ at lower temperatures \approx approximately 23,500

In the embryo of *Limulus* there is an early developmental period in which the heart is visible, contracting rhythmically, while the cardiac nervous system is still unformed, during this interval the heart rhythm is "myogenic" (Carlson and Meek, 1908) We have determined the relationship between temperature and frequency of heart beat during this period of "myogenic" rhythm, in order to compare the temperature effect with that in the heart ganglion of the adult. There seem to be clearly defined differences.

There is no reason to suppose that a difference in μ obtained in this way necessarily corresponds to or is diagnostic of myogenic as contrasted with intrinsically neurogenic processes. In so far as values of μ appear to be specific, and thus to correspond to physical realities

which may be utilized for purposes of classification and analysis,¹ it must be held that particular magnitudes of μ may reappear in all sorts of situations and do not pertain individually to particular types of function. But at the same time it is apparent that the occurrence of different systems of temperature characteristics for the two cases, embryonic heart and adult, is fully consistent with the idea that the respective essential controlling processes are unlike.

Such a result is of course not unexpected. The metabolic state of embryonic cells must differ materially from that of relatively greater dynamic stability enjoyed by the protoplasm of fully differentiated tissues. From this standpoint the apparently "irregular" variation of μ for rhythmic contraction in cultures of explanted chick myocardium (Murray, 1925-26) might be understood without reference to obscure regulation by the organism as a whole in order to account for greater uniformity in results when organs of intact animals are observed, even without appeal to structural conditions. When whole organisms are used it is possible to obtain modifications of temperature characteristics (μ), and these modifications appear to be specific (Crozier and Stier, 1924-25, *a*, 1925-26, *b*). It is entirely possible that the regularity of μ for comparable activities is partly determined by structural conditions in normal organs, such as permit of active control by definite "pace makers." Certain effects which seem to necessitate this view are discussed in a later section. It is perhaps of interest for this interpretation that the frequency of pulsation in the hearts of intact embryos may show considerable differences in μ among similar individuals (*cf* Crozier and Hubbs, 1924, and other cases), or in relation to age and other variables. The present observations show differences between individuals comparable to those experimentally induced in the breathing rhythm of the grasshopper (Crozier and Stier, 1924-25, *a*).

¹ Crozier, 1924-25, *a*, *b*, 1925-26, *b*. Crozier and Stier, 1924-25, *a*, 1925-26, *a*, *b*. Fries, 1926-27. It may be noted that change of μ coincident with the institution of neurogenic control would not necessarily prove diagnostic either, for we should require study of comparable developmental stages in the absence of nervous elements.

II

The heart of *Limulus* embryos within the egg envelopes becomes visible as a pulsating organ at the stage labelled *H* in accounts of the differentiation of the embryo (Kingsley, 1893, Kishinouye, 1893). For a period of about 6 days, or until Stage *K* (before the appearance of the telson), at laboratory temperature (Carlson and Meek, 1908), the activity of the heart continues to be visible, in the absence of nervous control.

The heart is not at any time particularly easy to see. This difficulty, together with expected variation in the effect of temperature, led us to practice special precautions in obtaining a large number of observations. The frequency of the heart beat is made visible by horizontal light of fair intensity, under which the cardiac tube appears as a delicate white ghost against the yellowish background of the substance of the embryo. In order to maintain the animal in a position suitable for observation, the egg membrane was punctured and a segment of it folded outward. This segment was fastened by white vaseline to a small glass block. The collapsed membrane holds the embryo in a relatively fixed position. Such preparations live in an apparently normal way for many days and continue to develop.

A number of glass blocks, carrying labelled embryos, are placed in a thin walled crystallising dish with sea water. Into this projects a microscope with paraffined objective used as an immersion lens. Dish and microscope are securely fastened to an iron frame supported on the rim of a large water thermostat. The vessel containing the embryos is submerged so that its water level is below that of the water in the thermostat. The temperature is read on a thermometer with enclosed stem, calibrated, and graduated to 0.05°.

In all such experiments difficulty is met in maintaining temperatures below that of the room. The latitude of fluctuation in frequency of contractions at constant temperature, necessitates a number of observations at uniform temperatures on each embryo. Yet it is required to change the temperature by a known small amount at intervals of about an hour.

To do this we constructed a thermostat from which heat could be abstracted by a SO₂ compression circuit. The coil and brine tank of a refrigerating unit were replaced by a considerable length of half inch Cu tubing coiled so as to form a helical shell within the wall of a 10 gallon glass tank. The motor operating the compressor was started through a relay actuated by a large mercury thermo-regulator². Sticking³ and sparking were obviated by using a Ni-steel needle to make contact with the fluctuating Hg surface and by having the relay of high

² For aid in this construction we are greatly indebted to Mr. H. V. Rivinius, refrigeration engineer of the Metropolitan Ice Co.

resistance (5000 ohms) ³ With adequate stirring, and felt insulation, this device enabled us to maintain for as long as desired any required temperature between 0° and that of the room. When an electric heater, constant or relay-controlled, is added to this arrangement, temperatures above that of the room are similarly obtainable, and the slight lag in temperature adjustment is still further reduced. The constancy of temperature within the body of the thermostat is then $\pm 0.001^\circ$. In the vessel carrying the objects to be studied the constancy is well within 0.01° , and this is improved by a cover.

The temperature is changed quickly by adding hot or cold water from a reservoir, excess in the thermostat being removed by a constant level device. The thermoregulator is readjusted by sucking Hg out of the regulator bulb, or by forcing more Hg into it from an accessory bulb. Finer adjustment is made by the screw-mounted contact needle. This operation required but a minute or so.

The thermostat is mounted upon a box, and a window is left in the insulation of the bottom. Through this window a beam of light is projected vertically upward, and may assist observation through the microscope or be employed in other ways.

The microscope, of ordinary type or a binocular with "Planktonsucher" objectives, is so mounted as to be movable over the observation chamber, but in the present experiments it was found that the glass blocks bearing embryos could easily be manipulated with a needle and in turn brought into position for the readings. These movements were found to be without influence upon the frequency of the heart beat, but some minutes were allowed to elapse before readings were taken. Lateral illumination was supplied by a small submerged electric lamp. It was easily shown that the light was without effect upon the frequency of contractions, but the general illumination was kept reduced as an aid to seeing the heart, thermometers, and thermoregulator being viewed by means of small accessory lamps.

III

When the frequency of the heart beat or of breathing movements is to be timed with precision in an intact animal it is necessary to avoid carefully the effects of concurrent movements of the body or appendages ⁴ In some instances it seems as if the execution of such movements is the cause of accelerations or retardations in the rhythm under observation, in others, it appears more probable that both disturbances have a common and simultaneous origin in the central nervous system. If it is desired to study intrinsic fluctuations of frequencies and to obtain temperature characteristics as precisely

³ Regulation may also be conveniently made by means of a system such as that described by Beaver and Beaver (1923)

⁴ Cf. Crozier and Stier, 1924-25, a

as possible, such deviations must be taken account of. In the *Limulus* embryo as in other embryos, there are evident from time to time "spontaneous" movements of body and legs. But it happens that in *Limulus* these movements, like the photokinetic movements of the legs which appear when the light intensity is suddenly changed, seem to have no influence whatever upon the sequence of the heart beats.

The frequency and the amplitude of the cardiac movements nevertheless go through a rather definite cycle, at constant temperature. This was ascertained by repeated observations on single individuals, since the data add nothing new to the theory of the case, they are not given here. The latitude of variation is slightly greater than 10 per cent of the mean frequency, and, as in some similar cases earlier described, is constant over the workable temperature range but varies from one individual to another. The latitude of variation seems quite unrelated, in general, to the relative rate of the process considered, and since it varies within pretty narrow limits for a variety of activities (5 to 10 per cent of the mean), it must be regarded as chiefly determined through some property of protoplasmic organization rather than by the specific process whose temperature characteristic is being measured. In a small number of known cases (Crozier and Stier, 1926-27, a) the latitude changes when μ differs on either side of a critical temperature, and in such cases a specific association must be assumed.

The slow developmental pace of the *Limulus* larva, together with the great resistance to asphyxiation (*cf* Kingsley, 1893, Newman, 1906, Redfield and Hurd, 1925) contribute to its suitability for our purpose. The majority of the individuals used were kept at 20°, when not subjected to temperature changes experimentally. No differences were seen in other embryos maintained at 4.5° for several weeks. It was possible in this way to have embryos of various stages of development available at one time. Reversing the course of the temperature changes gave observations in good agreement. Within certain limits duplicate "runs" on successive days also agreed well, but as a rule the frequency of heart beat changed after 1 or more days, although—so far as ascertained—without change of temperature characteristic. The total number of observations was 3400.

HEART OF LIMULUS EMBRYOS

IV

Sixteen series of readings, on fourteen different individuals, were well controlled by repeated check observations at the same tempera-

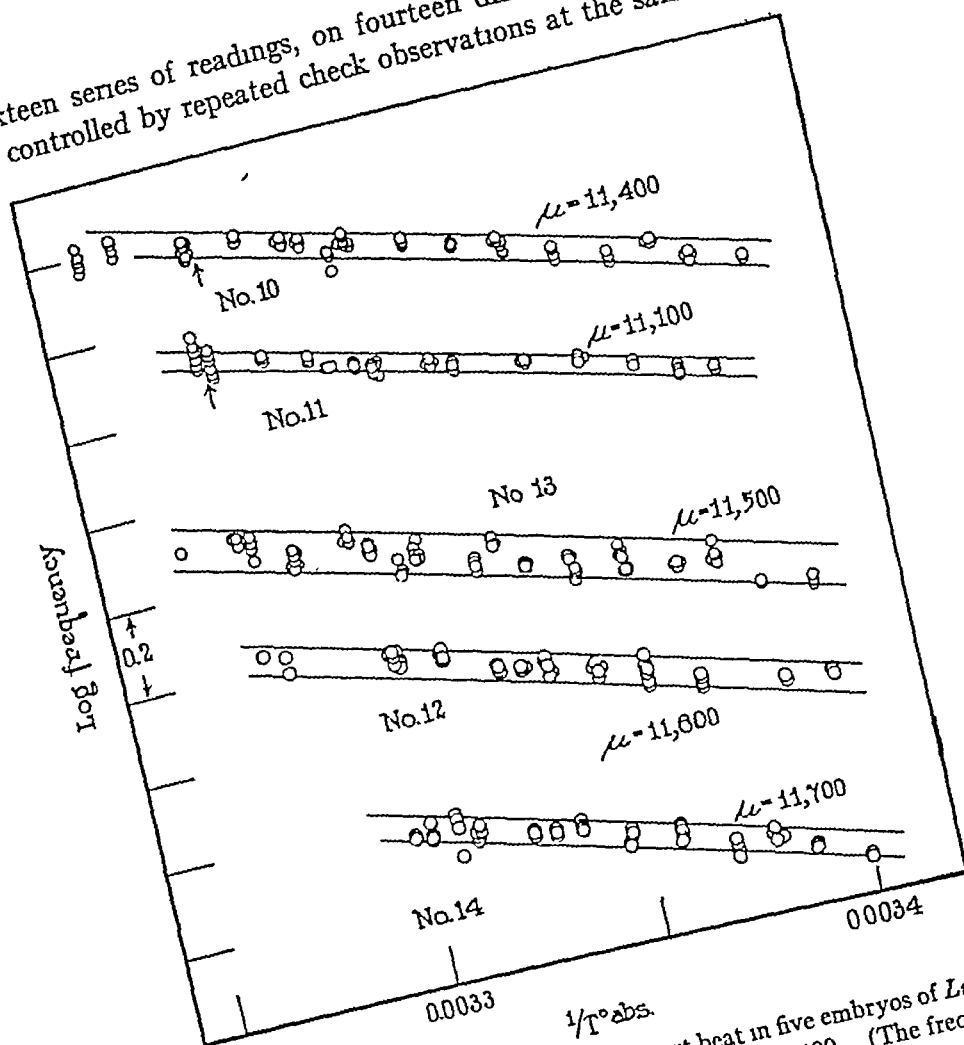


FIG 1 Observations on the frequency of heart beat in five embryos of *Limulus*, at temperatures between 20° and $35^{\circ} \pm$, for which $\mu = 11,400$ (The frequencies at constant temperature are very nearly the same, the frequency is taken as $100 \pm$ seconds for ten beats)

tures Of these individuals seven provide increments ranging from 11 000 to 12,280 (the latter value is probably too high), for these the average $\mu = 11,520 \pm 100$ Three series gave $\mu = 16,430 \pm 200$

Two gave $\mu = 20,000 \pm 100$, and two $\mu 25,500 \pm 300$. When data from any one animal are considered over a range of temperatures the lower value of μ pertains to the higher temperature interval (20° – 30° +), but one embryo gave $\mu = 11,000$ over the range 10° – 20° , and $\mu = 16,400$ occurs both in the range 10° – 20° and in 20° – 30° (with different embryos)

Above 30° – 34° the rate of increasing frequency of heart beat with elevation of temperature is very slight, most embryos of this

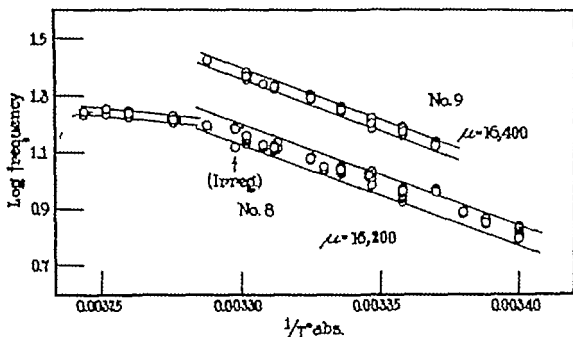


FIG 2 Data from two *Limulus* embryos for which $\mu = 16,300$ ($20^\circ \pm$ to $30^\circ \pm$), the rates for No. 9 have been multiplied by 1.59. The latitude of variation as seen also in Fig. 1, varies with the individual. Above $30^\circ \pm$ the frequency changes very little with increasing temperature the curve, within the region shown (No. 8) is perfectly reversible. This phenomenon has already been referred to as apparent in other cases (cf Crozier 1925-26, a)

age show slight but easily detected decreases in frequency of heart contraction, which are only very slowly reversible on return to temperatures below 30° . Other individuals do not exhibit this hysteresis, but above $30^\circ \pm$ the thermal increment is very small ($\mu = 5,000 \pm$) between 30° and 40.5° . This effect resembles that already noted in some other instances (Crozier, 1925-26, b), and is suggestive of the control of heart beat frequency by some purely physical condition, such as fluidity of substance or the saturation of some reactive sur

face which gives a mechanical limit to the maximum frequency of pulsation. In one case which is illustrated (Animal 2, Fig 5) no hysteresis was apparent on return to lower temperatures.

The temperatures for cessation of regular cardiac rhythm were 9° and 45° , above 40.5° the frequency of contraction decreases, at 44.4° the heart beat was still regular, but at 45.4° only an occasional beat was apparent. For the adult *Limulus* (Carlson, 1906) the thermal limits for contraction of the heart muscle are given as $0^{\circ} \pm$ and 32° ,

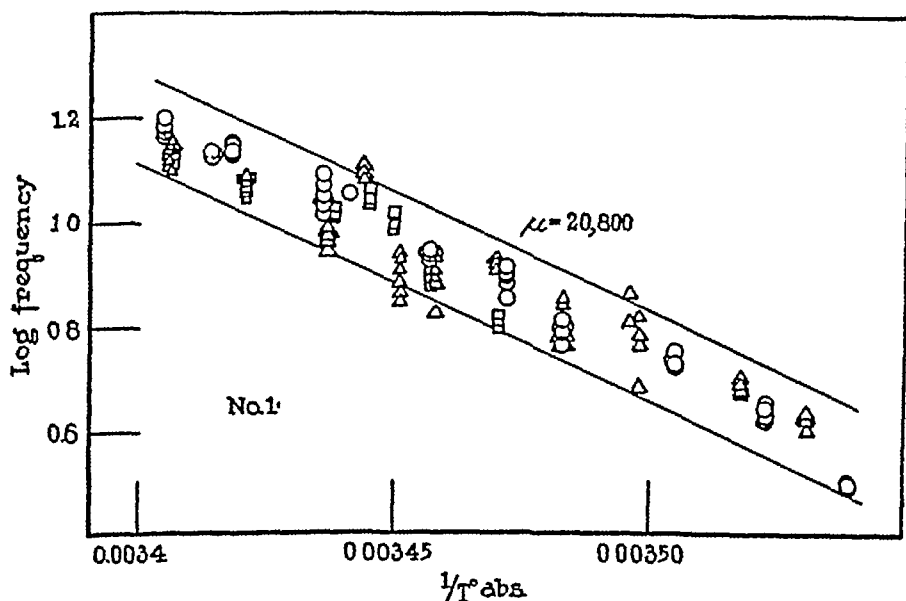


FIG 3 One individual gave data (three "runs" of observations) yielding $\mu = 20,800$ for frequency of cardiac rhythm, below 20°

with "heat rigor" appearing at 47° , for the cardiac ganglion and nerves, the limits of activity are 0° — and 42° – 43° . At the lower temperatures, beating was observed to become regular at 8.7° to 9.2° , below 8.7° , down to 5.9° , an occasional isolated beat was seen, on warming up to 8.8° to 9.0° , regular contractions were uniformly observed. It is worth noting that these thermal limits, determined from observations upon a large number of embryos, were found to be the same in embryos kept for 15 days at 4.0° as in those maintained at room temperature or used in warming or cooling experi-

ments. This agrees with the essence of Mayer's (1914) findings upon the adult *Limulus* and points to the conclusion that in this instance the thermal effects depend upon the composition of the animal, rather than upon thermal adaptation.

Between the extremes of temperature which limit the exhibition of regular rhythm (9° , 40.5°) the following are found to be critical temperatures, in the sense (Crozier, 1925-26, a) that abrupt change or irregularity may there appear in the curve relating frequency to

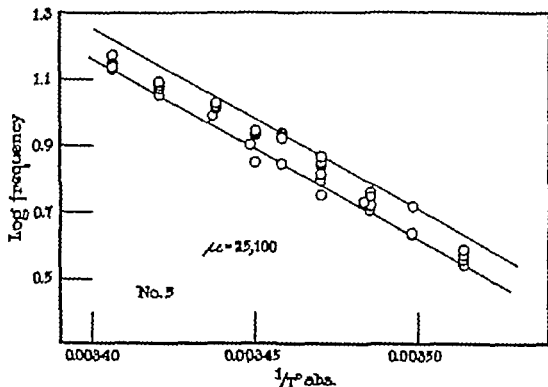


FIG 4 *Limulus* Embryo 5 gave $\mu = 25,100$, below 20°

temperature 20° , 27° , 30° to 34.5° . We purposely avoided the possible influence of the 'break' at $20^{\circ} \pm$ by largely working below or above this temperature, with different embryos. One individual (No. 2, Fig. 5) was found by repeated runs of observations to exhibit a sharp "break" in the curve of heart beat frequency at 20° - 21° of such a character that a change in frequency accompanied a change of temperature characteristic. This is the first instance of the kind which we have been able to study carefully. The probable existence of such cases has earlier been mentioned (Crozier, 1925-26, b), they are of particular interest for the theory of critical temperatures.

HEART OF LIMULUS EMBRYOS

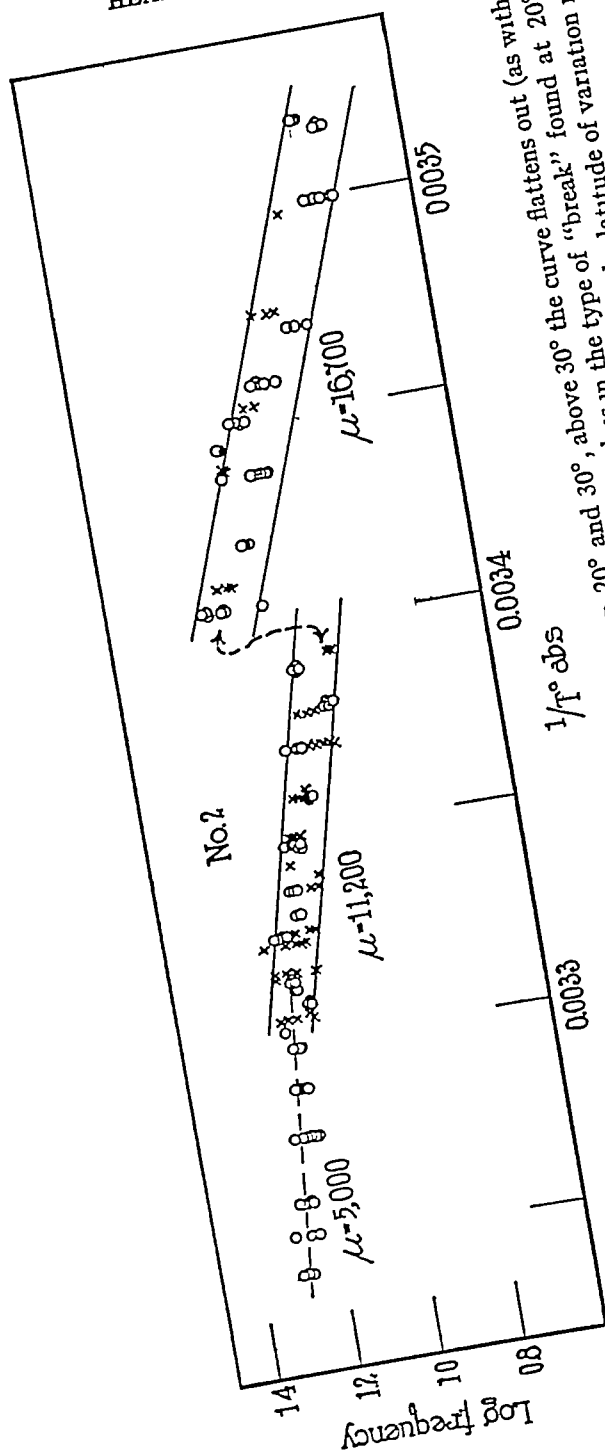


FIG 5 Embryo 1 gave $\mu = 16,700$ below 20° , $\mu = 11,200$ between 20° and 30° , above 30° the curve flattens out (as with No 8, Fig 2, and in other instances not plotted). The particular interest of this graph is in the type of "break" found at 20° (see text). Two series of observations are distinguished by symbols, in the second series (crosses) the latitude of variation is less, below 20° , than in the first series

V

The temperature characteristics for frequency of cardiac rhythm in *Limulus* embryos thus appear as definite and recurrent quantities. The fact that two individuals apparently similar may yield quite different magnitudes of μ simply means, we take it, that the two pace maker cells or cell groups in these hearts have slightly different metabolic adjustments, or that the pace maker groups are different. If this were correct we might reasonably expect to alter μ experimentally in a more or less predictable way. This we did not attempt in the present observations although it has been accomplished in other instances (Crozier and Stier, 1925-26, b). It would also be predicted that the various values of μ obtained should show certain interrelationships. Thus the common association of the values 11,300 and 16,200 in respiratory and other processes (Crozier, 1924-25, b) finds rational application in the present case.

For the heart of adult *Limulus* the characteristic μ , so far as can be ascertained, is about 12,200, in several individuals (data from Garrey, 1920-21, a, b, cf Crozier, 1924-25, a) $\mu = 23,500$ below 15° . With one exception, which is not intrinsically of great weight, this value of μ does not appear in connection with the embryonic heart. The data upon adult heart rates came from experiments in which the temperature of the cardiac ganglion alone was varied, and the μ obtained agrees quantitatively with that for a number of other instances among arthropods in which central nervous control may be assumed (Crozier, 1924-25, a, Crozier and Stier, 1925-26, a, Fries, 1926-27). The increments apparent in the observations on the embryonic hearts, however, are of frequent occurrence in data on the heart rhythms of molluscs and vertebrates (cf Crozier 1925-26, b). This sort of result points definitely to different chemical control of heart pulsation in embryo and in adult *Limulus*, and to the relative diversity of the pace-making control in the developing embryos. (If adult *Limulus* were to be used, the possibility of myogenic effects might have to be reckoned with, in addition to the neurogenic automatism, if the temperature of the whole organism were varied, cf Hoshino, 1925.)

The possibility of diverse pace making processes in the hearts of different individual embryos, and thus in different but functionally

analogous cells, is important for the understanding of thermal effects in isolated heart preparations and in cultures of developing myocardium. To this there must be added the recognition of sources of confusion which may result from the fluctuation of controlling circumstances within single cells, of which at least two kinds of disturbing effects can reasonably be suggested. The behavior of the "accessory hearts" of *Notonecta* is particularly significant in this connection (Crozier and Stier, 1926-27, *b*). At the moment we wish to deal particularly with the consequences of the occurrence in a single pulsating structural mass of a diversity of possible pace-making elements (*cf.*, for the chick heart, Cohn, 1925). Murray (1925-26) found that the apparent temperature characteristics for frequency of pulsation in cultured explants of chick myocardium failed to show uniformity, and failed to be grouped about detectable modal values. In such preparations there must exist at any moment a number of possible pace makers. The net result of their fluctuating control would be expected to obscure or to blur the influence of any one, since it is fair to assume that their respective inner metabolic states might be differently adjusted. In heart cell cultures the controlling influence of the intrinsically faster beating component of compound masses has been demonstrated experimentally by Olivio (1926), and this appears indeed to be a general condition (*cf.* Mayer, 1911, Crozier, 1916).

The sort of situation, therefore, which we believe to exist in pulsating heart cell cultures is one in which a number of distinct "pace-making" cells or cell groups are present in each pulsating mass. The intrinsic frequency of initiation of rhythmic contraction is supposed to differ among these pace makers. If one pace maker definitely possesses a much faster rhythm than the others, its effect is uniformly apparent. But if two or more pace makers have nearly the same intrinsic frequencies, but are metabolically different, so that each exhibits a characteristic relation to temperature, their several influences upon the gross sequence of pulsations should interpenetrate, at one moment pace maker *A*, at another instant, before *A* starts again, pace maker *B* is in control. One consequence of this kind of effect may be tested immediately. The latitude of variation, expressed as a percentage of the mean pulsation-frequency at each

temperature, should not be constant if pace makers *A* and *B* have different temperature characteristics. This is precisely the situation disclosed in several of Murray's (1925-26) figures. Therefore temperature characteristics deduced from such data *en mass* must be regarded as without specific significance.

Three corollaries are at once deducible (1) The situation here pictured and tested differs from that (Crozier, 1924-25, *a*) in which it is supposed that the slowest process of a catenary series of catalyzed transformations dictates the speed and temperature characteristic for the velocity of formation of the end result. In the present case the swiftest pace maker determines the maximum frequency of rhythmic contraction. Therefore we may expect to find cases in which the "mean" temperature characteristic is increased at higher temperatures. The realization of this possibility is suggested in several figures given by Murray (1925-26, Figs. 1, 2, 3). One effect of this would be to bring about instances in which the log latitude of variation increases with increasing temperature as well as others in which the change is in reverse direction. These differences appear in Murray's figures.

(2) Of greater interest is the corollary that if our assumed pace makers *A* and *B*, respectively generating the most frequent and the least frequent contractions among the group capable of acting as pace makers at all, should have the same temperature characteristic, then the logarithmic latitude of variation should be constant, and in such cases the μ deduced should correspond well with a value found in homologous instances. In the figures given by Murray (1925-26, Figs. 1, 2, 3) we find, for cases meeting this requirement, $\mu = 8,000$, $\mu = 11,000$, and $\mu = 16,100$, these compare well with values commonly recognized in heart rate measurements (Crozier, 1925-26, *b*).

(3) For cases in which the latitude of variation is inconstant, the slopes of the lines fitting the extreme variates on the semilog plots should be straight and should provide approximate μ values characteristic of the limiting pace makers. And we should expect these to compare favorably with temperature characteristics encountered elsewhere. We have treated in this way the plots given in Murray's Figs. 1, 2, 3. The result in one case is reproduced in Fig. 6. The

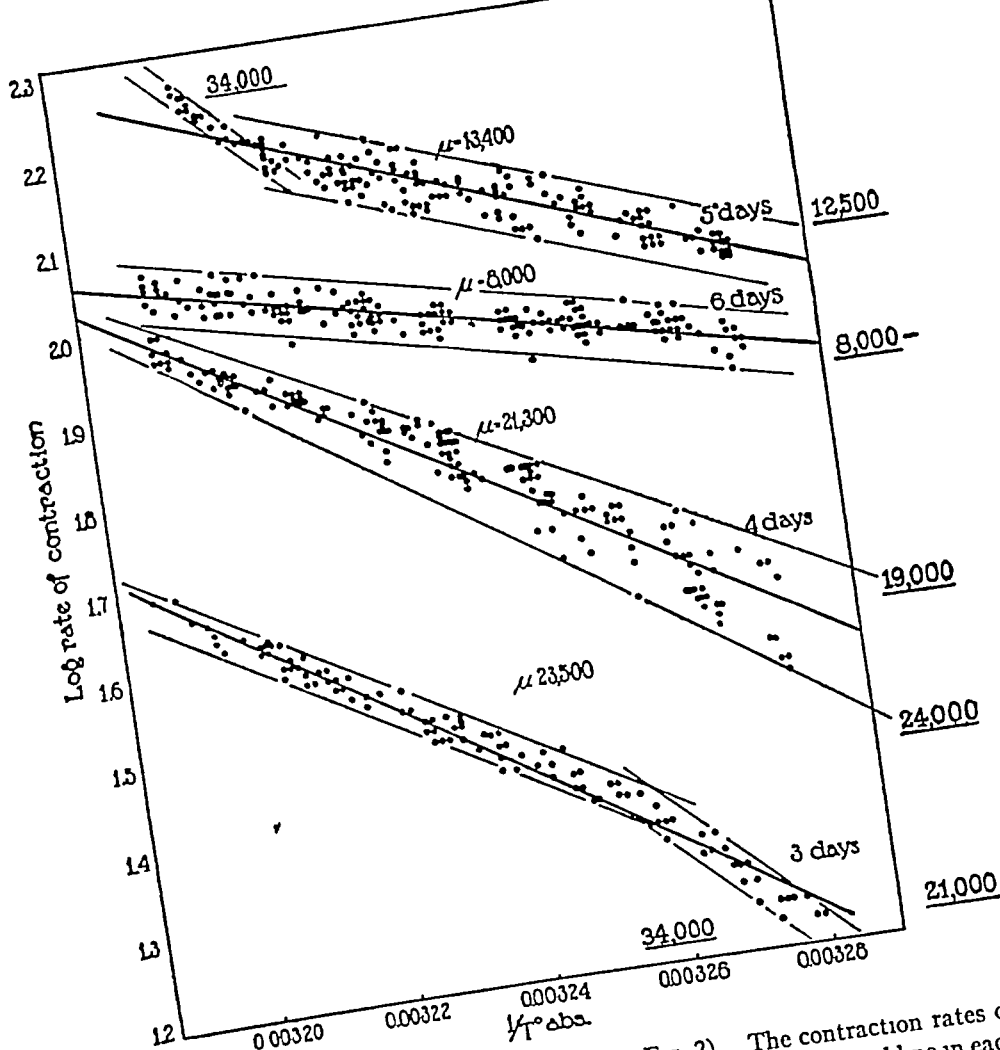


FIG 6 Reproduced from Murray (1925-26, Fig 2) The contraction rates of auricular fragments from embryos of the ages indicated. The central line in each plot is that originally given by Murray. To these lines there have been added marginal lines fitting the extreme variates. When attention is given to the latitude of variation it is obviously necessary to deduce values of the temperature characteristics somewhat different from those originally given. Values obtained from the marginal lines are indicated with underscoring. The nature of "breaks" in the uppermost and the lowermost graphs are fairly clear. When the log latitude of variation is constant, values of μ are gotten ("6 days," etc.) which are already well known in other situations. When the latitude of variation changes continuously on the semilog plot the marginal rates are regarded (see text) as due to the operation of diverse pace makers. We consider that the recognition of these sources of confusion in curve fitting is sufficient to remove the force of the contention that temperature characteristics vary at random in this case.

characteristics obtained are noted in the figure, and the result may certainly be taken to agree with the expectation

The effect of two such limiting pace makers could be imitated by combining the observations from two individuals (*Limulus*) in which μ for frequency of heart beat is different but the rates at given temperature approximately the same. It may be suggested that precisely this condition may appear if one were to measure the frequencies of contraction in the heart of an Ascidian, without reference to the places of origin of the individual beats, or, perhaps better, in a medusa deprived of all but several rhopalia.

These considerations do not completely account for the sources of complexity probably present when such an object as a heart cell culture is studied in this way. The indications already obtained however, show why in these analyses we have continuously insisted (1) upon the errors which may be involved in the process of averaging rates or frequencies of vital processes in different individuals, or even in the same individual at different times, and (2) upon the ribbon form of significant plottings. There is to be added the further type of difficulty entering when a break occurs in the curve relating frequency or rate to temperature, should this sort of change be present in the activity of one pace maker, absent in others, the logarithmic latitude of variation must again change if the curve for this pace maker falls outside the limits set by the activities of other concurrently effective pace makers. Such a break, furthermore, may or may not be accompanied by an abrupt change of frequency, and changes of frequency may occur without change of temperature characteristic. These are not imaginary situations (*cf* Crozier, 1925-26, *b*, Crozier and Stier, 1924-25, *b* 1925-26, *b*). It seems to us inherently probable that disturbances of these types are likely to be encountered with greater frequency in objects such as isolated cell masses in culture than in connection with organs of intact animals, although we may also suggest their probable occurrence in the heart rhythms of embryos. The plottings given by Murray (1925-26) contain features suggestive in this respect, which we venture to predict will find explanation in further studies of embryonic heart rhythm.

There is one general aspect of this whole matter which requires brief additional comment. Murray (1925-26) has suggested that

the essential difference between the gross results of his observations and those in cases where intact organisms have been employed lies in the operation of some regulatory property of the complete organism. Since there is a possibility of vagueness in the understanding of such

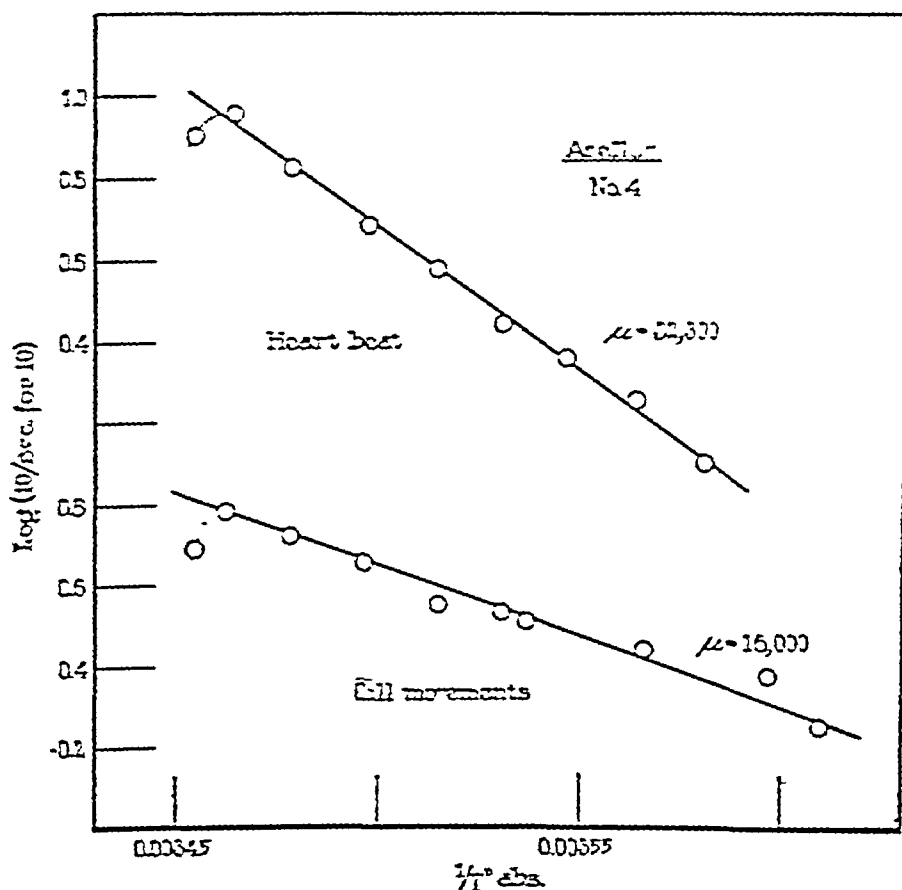


FIG. 7. Simultaneous determinations of frequency of gill movements and of heart beat in *Asellus* show that the temperature characteristic is not the same for the two activities. This disproves determination of μ by the organism as a whole.

a suggestion we may point out that it may be understood in two ways open to test. The 'organization factor' might pertain simply to the heart or other structure immediately implicated in the observations, or it might be taken as a feature of the organism as a whole.

The former effect can and must be granted at once as an obvious truism, in the sense that the structure of a heart, for example, permits control by a definite localized pace maker. The latter view can be tested by determining simultaneously the temperature characteristics for two or more different activities in the same individual. We have previously made such experiments. The frequencies of heart beat and of respiratory movements in the same individual arthropod, synchronously determined, do not vary together and do not have the same temperature characteristics. This holds also for embryonic *Limulus*, the frequencies of gill movements providing increments quite different from those here obtained for the hearts (cf Crozier and Stier, in a subsequent paper). For the moment we may illustrate the point by means of data from experiments with *Asellus* (Fig 7). Therefore a general control by the whole organism is excluded. The results of these experiments will be detailed in another place. They are patently significant for the theory that a specific thermal increment has a particulate locus.

SUMMARY

Temperature characteristics for frequency of myogenic heart beat in *Limulus* embryos, before the onset of nervous control of the heart, were found to be 11,500, 16,400, 20,000, 25,500. The two first values are the best established. The different values pertain to the hearts of different individuals outwardly similar, and to the hearts of single embryos in different parts of the temperature range. These values differ from that known in connection with the control of the heart beat through the cardiac ganglion. The occurrence of critical temperatures, also, is not the same in all embryos. These facts are employed in a discussion of temperature relations in pulsating explants of chick myocardium.

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GEOTROPIC ORIENTATION OF YOUNG RATS

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I

In describing the geotropic conduct of young rats (*Rattus norvegicus*)¹ we have stressed the fact that to obtain data suitable for quantitative treatment of the gravitational orientation it is necessary to employ individuals of the closest possible similarity. This has meant, in practice, employing litter mates from lines long inbred, under external conditions as nearly uniform as possible. The result has demonstrated that, with these precautions taken, it is possible to formulate precisely the connection between the intensity of excitation and the extent of the geotropic orientation. On a creeping plane inclined at angle α to the horizontal, and in which the effective gravitational component is therefore proportional to $\sin \alpha$, the orientation path is one defined by an angle θ on the plane, such that $\theta = K \log \sin \alpha - C$. The precision of the orientation increases according to the same equation. In addition, $-\cos \theta = K \sin \alpha$. The *speed* of progression bears similar relations to the angle α .

It was proposed to interpret these results as signifying that on an inclined plane the rat orients upward until the difference between the work done by the legs of the two sides is reduced to a certain (constant) fraction of the total. It is possible to entertain this view because the differential postures of the legs encourage it, and because they are extended in the plane of creeping. It is supported by the effects of increasing the mass lifted during creeping, as by attaching weights to the base of the animal's tail. This conception of the geotropic excitation controlling the amount of orientation as a proprioceptive matter is strengthened by further findings in this laboratory regarding the

¹ Crozier and Pincus 1926 1926-27 a b Pincus, 1926-27

orientation of molluscs Mr T J B Stier has also observed an identical type of orientation in newts (*Notophthalmus*), with the additional important fact that when the sign of orientation is reversed, and the animal then becomes *positively* geotropic, the angle θ is the same as in the more usual geonegative orientation

An immediate corollary to these findings in rats of one type (*R norvegicus*) was the possibility that certain genetic or specific differences might find expression by means of the constants in the equations for geotropic behavior This deduction we now propose to illustrate As material for this purpose we chose a strain of the roof rat (*Rattus rattus*) Individuals of the proper age, 13 days, were very kindly

TABLE I

The angles of upward orientation (θ) during creeping of young *R rattus* upon a plane inclined at angle α to the horizontal The values of θ are each the mean of fifteen determinations, three on each of five rats in one litter

α	θ	P E θ , as per cent θ
		<i>per cent</i>
10°	27 0°	8 74
15°	37 1°	5 78
20°	48 1°	3 68
30°	61 9°	2 34
40°	71 0°	1 88
50°	80 1°	1 72
60°	83 5°	1 06

placed at our disposal by Dr H W Feldman of the Bussey Institution We are greatly indebted to Professor W E Castle, and to Dr Feldman, for this and other like assistance

In this rat the geotropic influence could be expected to be more pronounced Aside from the matter of its persistence into adult life, the relatively greater lengths of the legs, and the somewhat less body weight, were each expected to play a part in modifying the constants of the equations for geotropism

II

Five members of one litter, 13 days after birth, were employed in securing the final series of observations collected in Table I The

experiments were made in a dark room, with temperature 20°–23°, following the technique outlined in previous papers¹

Within the limits of the probable errors of the means the measured values of θ adhere quite precisely to the relationship already estab-

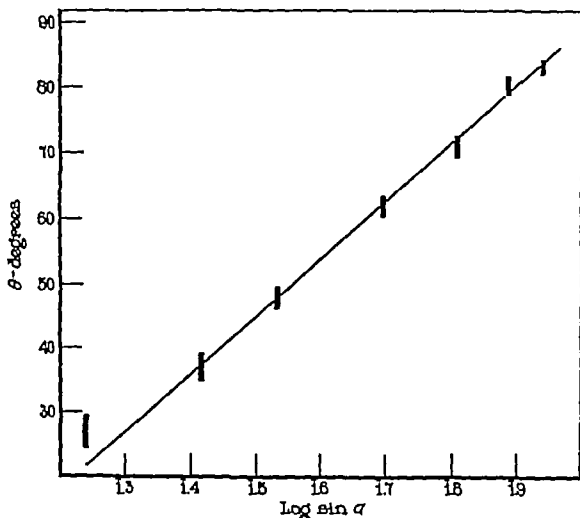


FIG 1 The extent of upward orientation (θ) on a plane inclined at angle α to the horizontal is linearly related to $\log \sin \alpha$ during geotropic creeping of young *R. rattus*. Each observed angle of orientation is plotted as a bar of which the height = 2 P.E. The departure of the observed angle at the lowest inclination (10°) is referred to in the text.

lished¹ for the Norway rat, as Fig 1 shows, and they thus provide an independent confirmation of it. The precision of orientation decreases linearly as $\log \sin \alpha$ increases (Fig 2). The observations at the lowermost magnitude of α are necessarily very variable, because the slight geotropic stimulation does not sufficiently inhibit movements orig-

inating in other ways. The fact that here again, as with the Norway rat, $\cos \theta$ decreases linearly as $\sin \alpha$ increases, is made evident in Fig. 3.

To compare these results with those given by the species first used it is necessary to obtain the constants in the several equations. This is most conveniently done from Fig. 2. For the roof rat the "ideal threshold" for geotropic orientation is 3.5° [$\cos \theta = 1$], as compared with 6.5° for *norvegicus*, and 90° orientation is obtained at a slightly lower angle (67.2°) than in *norvegicus* (70.0°). This is in accord with the expectation that the "geotropic sensitivity" of the roof rat should be greater. The equation describing the orientation (Fig. 3) is

$$1 - \cos \theta = K \sin \alpha - M \quad (1)$$

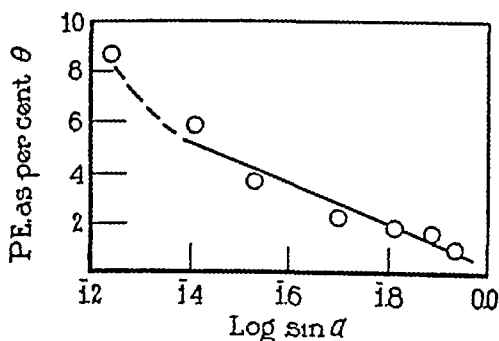


FIG. 2. The variability of the measured angle of orientation (θ) decreases almost linearly in proportion to $\log \sin \alpha$. At the lowest inclination the variability is disproportionately large, because the "threshold" presumably differs from moment to moment.

For *R. norvegicus*, of the type used in our previous experiments,¹

$$\begin{aligned} K &= 1.206 \\ M &= 0.113 \end{aligned}$$

For *R. rattus*,

$$\begin{aligned} K &= 1.18 \\ M &= 0.06 \end{aligned}$$

In terms of Fig. 1,

$$\theta = K' \log \sin \alpha - C \quad (2)$$

For *R. norvegicus*,

$$\begin{aligned} K' &= 100 \\ C &= 1.998 \end{aligned}$$

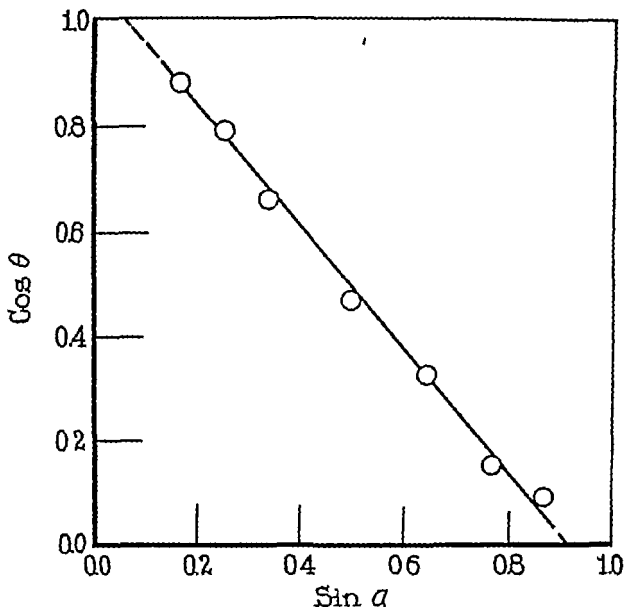


FIG. 3. As with *R. norvegicus* (Crozier and Pincus 1926-27 a b), in *R. rattus* the relationship between $\cos \theta$ and $\sin \alpha$ is rectilinear.

For *R. rattus*

$$\begin{aligned} K &= 90.4 \\ C &= 1.933 \end{aligned}$$

III

Our sole purpose in recording the outcome of the present experiments is to demonstrate that the methods employed are not only capable of yielding statistically significant results in terms of an intelligible mechanism, but also of providing a method for the precise characterization of behavior differences within genetically uniform strains. It is not too much to hope that the obvious development and application of this view-point may lead to results significant for inheritance studies. Certain aspects of this matter we expect shortly to have in hand.

IV

SUMMARY

The geotropic orientation of *Rattus rattus* (roof rat) obeys the equations previously found applicable for *Rattus norvegicus*. The former is more sensitive, geotropically, and the numerical values of the constants in the equations for the two forms are found to differ significantly. Certain consequences of this difference are pointed out.

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GEOTROPIC CREEPING OF YOUNG RATS

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(Accepted for publication December 2, 1926)

I

The geotropic conduct of young rats has been discussed in a previous paper (Crozier and Pincus, 1926-27) with special reference to the angle of orientation upon an inclined plane. It was found that the angle of orientation (θ) is directly proportional to the logarithm of the gravitational component ($g \sin \alpha$) in the creeping plane. This is explicable as the result of the distribution of the pull of the animal's weight upon the legs of the two sides of the body during progression, upward orientation being the result of the "pull" of the legs on one side and the upward "push" of the legs on the other side, when orientation is attained, the ratio of the tensions on the legs of the opposite sides is regarded as constant and the difference between these tensions as a constant fraction of the total downward pull.

To examine further the nature of the geotropic conduct of young rats, observations on the speed of upward creeping were undertaken. Cole (1925-27) has discussed similar observations on *Helix*, he concludes that the speed of movement, after orientation has been attained, varies as $\sin \alpha$. But, as has been pointed out already (Crozier and Pincus, 1926-27), in these experiments the speed measured was that of vertical ascension, and no correction was made for the changes of θ at the different angles of inclination, such changes occur in the orientation of gasteropods. Since, at lower angles of inclination of a creeping plane (15° - 70°) the animal moves at an angle (θ), it is necessary to multiply the time of upward creeping by the sine of the angle of orientation (θ) in order that the amount of time actually necessary to cover a constant distance may be dealt with at each angle of inclination (cf Fig 1). In terms of Fig 1, the rate of creeping is given by the

fraction $\frac{AB}{t \sin \theta}$, where $AB = 32$ cm, and t is time in seconds. The required rates are therefore proportional to $\frac{1}{t \sin \theta}$.

II

The rats used in these experiments were 13 to 14 days of age. As in the previous experiments, only animals with unopened eyes were used. To insure uniformity the animals employed were of the same age (litter mates), of known genetic constitution, and of the same weight. It was soon found that the animals show periods of activity

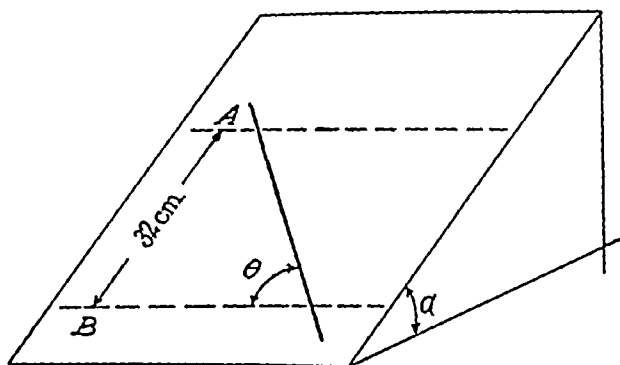


FIG 1 On a plane inclined at angle α the path of geotropic orientation is (solid line) at angle θ , the time was measured for creeping the distance 32 cm as indicated. The rate of progression is then obtained as $(AB)/(t \sin \theta)$

followed by periods of inactivity, the latter being possibly due to or influenced by fatigue. Care was therefore taken to use only active individuals, and between tests each animal was allowed to rest for 20 to 30 minutes.

Observations were made in a dark room under red light of low intensity, at a temperature of 23° – 25°C . The animals were placed on a creeping plane of wood covered by fine meshed copper wire. A distance of 32 cm was marked off on the creeping plane with white chalk, and by means of a stop-watch the time was taken for the animal to creep from one white line to another.

At least ten runs were made at each angle of inclination, and at 15° and 20° inclinations, where the speed is more variable, twenty runs were recorded

III

Table I contains (a) the times for creeping between two lines 32 cm apart (*AB* in Fig 1) as obtained for various angles of inclination from 15°–60°, and (b) the corrected rates secured by multiplying the observed rates by the sine of the angle of orientation (θ) and taking the reciprocals. No records for inclination above 60° were taken because these young animals have not the muscular equipment

TABLE I

Angles of Orientation and Rates of Creeping at Different Inclinations of the Creeping Plane.

Angle of inclination (α)	Time to creep 32 cm. vertically	Probable error as per cent of the mean	Angle of orientation (θ)	Rates of creeping $\frac{10}{t \sin \theta}$
15	6.73	3.14	37.4	2.442
20	5.42	2.94	44.5	2.636
25*	4.73	2.82	52.9	2.654
30	4.14	2.23	57.4	2.872
35	3.61	2.42	64.0	3.082
40	3.40	1.84	69.8	3.139
50	3.10	1.63	77.9	3.300
60	2.95	1.05	84.7	3.407

necessary for uniform response to the more intense geotropic excitation of the higher angles of inclination. The speed of creeping, like the angle of orientation, varies directly as the logarithm of the angle of inclination (Fig 2). Therefore the speed of creeping should be directly proportional to the angle of orientation (θ), and Fig 3 shows that this is the case.

In a preceding paper (Crozier and Pincus, 1926–27) it has been shown that the *precision* of upwardly directed movement increases as the angle of inclination increases. That is, the reduction of variability (V) in the measurements of θ is proportional to the logarithm of the gravitational stimulus

$$-V = K \log \sin \alpha.$$

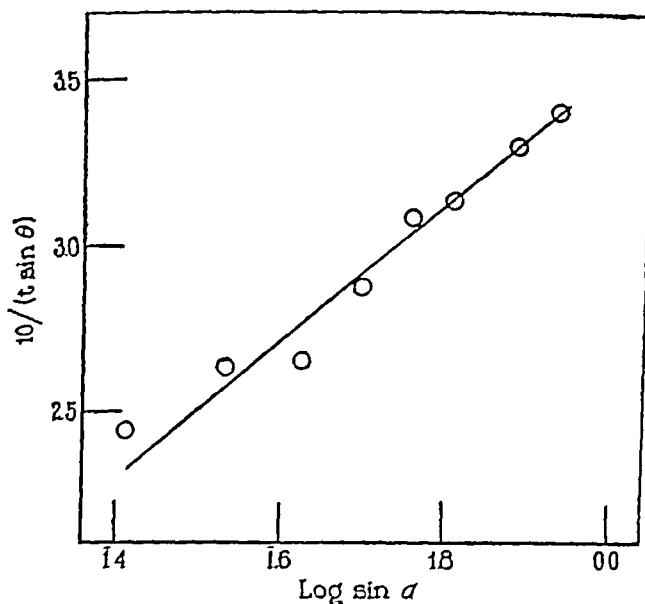


FIG 2 The rate of creeping expressed as $10/(t \times \sin \theta)$ is plotted against the logarithm of the active gravity component. A direct proportionality is observed, the spread of the points at the lower inclinations indicating the increase in variation with lower intensities of stimulation.

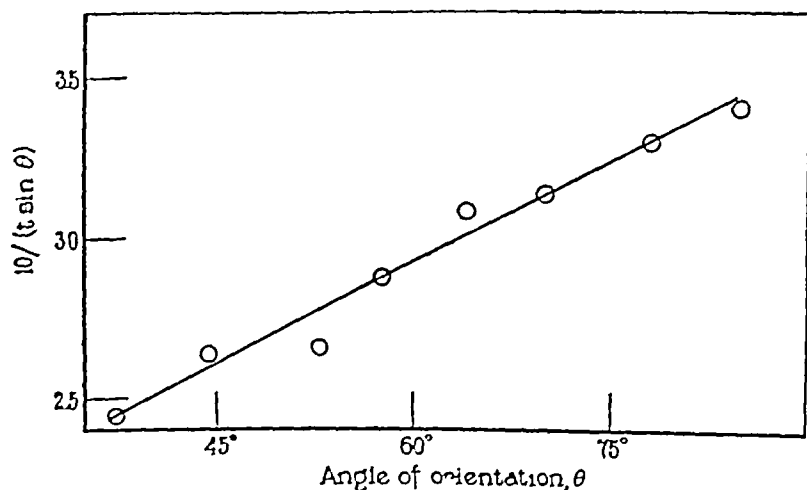


FIG 3 The rate of creeping is plotted against the angle of orientation (θ). Since both are directly proportional to the logarithm of the active gravity component it follows that they should be directly proportional. This is the case, and a check is had upon the relation expressed in Fig 2.

This relation is plotted in Fig. 4 for the speed of creeping. The speed of creeping is a much more complicated thing than the simple geotropic orientation, and is influenced by unanalysed fatigue effects and by cycles of activity. Furthermore, only ten observations were taken at each inclination. Nevertheless, it is apparent that the variability in speed of creeping is less at higher angles of inclination than at the lower angles, and that the relationship is linear, as in the case of the amount of upward orientation.

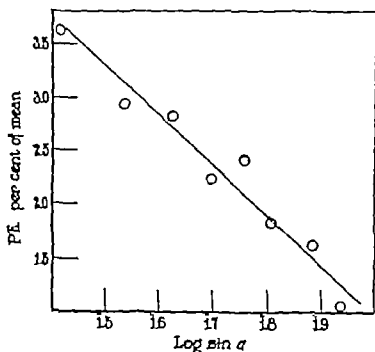


FIG. 4. The 'coefficient of variation' expressed by the probable error as per cent of the mean, is plotted against the logarithm of the active gravity component. The result indicates that the variability in the rate of creeping decreases as the angle of inclination (α) is increased, and in the same manner as the variability of the extent of upward orientation (Crozier and Pincus 1926-27).

IV

When weights are attached at the base of the tail of a young rat creeping on an inclined plane the angle of orientation increases approximately as the logarithm of the added weight (Crozier and Pincus, 1926-27). To test this relation further, the speed of creeping with attached weights was measured at two angles of inclination, 15° and 20° . Weights of 1.6, 2.6, 5.2, 7.0, and 9.8 gm. were used.

The angle of orientation (θ) with attached weights was measured as well as the rate of creeping, and correction of the rate was made by multiplying the observed times by $\sin \theta$, as before. The results are given in Table II. Fig 5 gives the corrected rate plotted against the logarithm of the added weight and indicates a direct proportionality for the 20° inclination, at the 15° inclination the plot is apparently curvilinear. However, when, as in Fig 6, the corrected rates are plotted against the angles of orientation (θ) the direct proportionality observed indicates that the curvilinear distribution in Fig 5 for 15° is accidental.

The significance of these data lies in the fact that they demonstrate the proportionality of geotropic response to the logarithm of the ac-

TABLE II

Angle of inclination	15						20°					
Attached weight (gm)	0	1 6	2 6	5 2	7 0	9 7	0	1 6	2 6	5 2	7 0	9 7
Angle of orientation (θ)	37 35	44 0	51 3	67 0	72 5	84 3	47 95	51 0	62 0	78 1	80 3	90 0
Time of creeping (seconds)	6 63	5 27	4 50	3 54	3 15	2 77	5 07	4 57	3 64	3 12	2 85	2 65
$10/t \sin \theta$	2 477	2 734	2 849	3 071	3 332	3 628	2 658	2 816	3 115	3 276	3 562	3 773

tive gravitational component, rather than to the gravitational intensity directly. They give further confirmation of the hypothesis that the geotropic responses are the result of the pull of the animal's weight on the legs of opposite sides. This makes it unnecessary, or indeed impossible, to account for the orientations in terms of the pull of the head upon the neck muscles. It may be emphasized that as the weight of the attached load is increased, or as the angle of inclination of the creeping plane is increased, the legs are actually further extended. The angle of orientation, however, seems to be determined by the difference in effective pull on the legs of the two sides, such that, diagrammatically, at orientation $(x - y) \cos \theta = KMg \sin \alpha$, where x and y represent the lever radii of the legs on the "down" and "up" sides

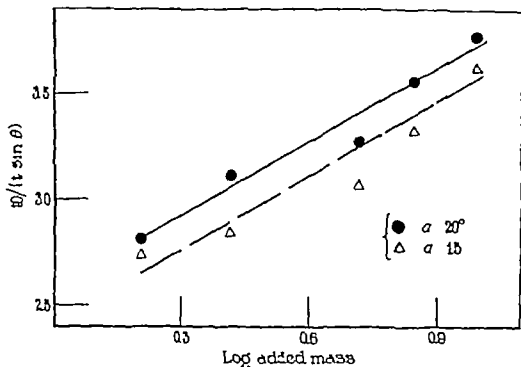


FIG 5 The rate of creeping with attached weights at two angles of inclination (15° and 20°) is plotted against the logarithm of the attached weight. At 20° the result indicates a direct proportionality. At 15° the result is apparently curvilinear, but this may be due to fortuitous variations in θ .

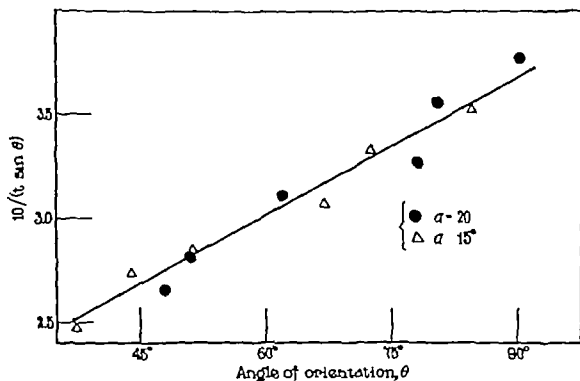


FIG 6 The rate of creeping with attached weights at two angles of inclination (15° and 20°) is plotted against the observed angles of orientation at these angles of inclination. The result shows a direct proportionality; the curvilinear relation at 15° as apparently indicated in Fig 5 is not detectable.

of the body, respectively. If $KMg \sin \alpha$ be increased by adding a load to the rat's tail, K remaining a constant and M being the mass lifted, $(x - y) \cos \theta$ must increase, hence $(x' - y)$ must become larger (since θ is increased), therefore the legs on the "up" side are further extended, relatively, than in the absence of added weight, this is a fact of observation. Thus the increase in θ , with α constant, when a weight is added, results from the extension of the limbs by the added load, since the "upper" or y limb is stretched and thus more extended. The speed of creeping is influenced in exactly the same way as the extent of orientation.

SUMMARY

The rate of upward creeping in negatively geotropic rats aged 13 to 14 days is a function of the gravitational stimulus. The rate of upward movement on the creeping plane, like the angle of orientation, is directly proportional to the logarithm of the gravity component. The variability in the speed of creeping decreases in proportion to the logarithm of the gravitational effect. When weights are attached to the animals' tails the rate of upward creeping varies almost directly as the logarithm of the attached weight, and the speed of creeping is still proportional to the angle of upward orientation.

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POTENTIOMETRIC STUDIES ON INTRACELLULAR pH VALUES OF SINGLE FUNDULUS EGG CELLS

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Studies on internal reactions of cells as well as permeability of cells to acids and alkalis have usually been concerned with changes in the color of intravital indicators, introduced or taken up by the cell, as well as changes in naturally occurring cellular pigments (1-6) Death and functional changes within cells have also been used as criteria of penetration of acids and other compounds into certain specialized types of cells (2, 7) From the standpoint of an accurate quantitative treatment of intracellular pH changes in such experiments the usual errors and limitations of colorimetric as well as of mortality methods are ever present and seem to allow but limited experimental procedures The present paper is based upon an attempt to study the intracellular reaction (pH) in single egg cells of a marine fish, *Fundulus heteroclitus*, by means of a micro hydrogen electrode and vessel originally designed for the determination of the pH values of minute quantities of insect blood (8) With suitable modifications of the electrode vessel it has been found possible to make three separate determinations on a single *Fundulus* egg cell.

Method

The micro hydrogen electrode and vessel used in these experiments were as originally described (8) with the exception of a capillary vessel modified so that a drop as small as 0.01 cc. could be conveniently handled. Both fertilized and unfertilized eggs of *Fundulus heteroclitus* obtained at Woods Hole Massachusetts were used. All eggs were taken directly from the female by 'stripping' them into the solution contained in finger bowls Eggs from each animal were kept and tested separately In experiments where rates of penetration of acid into the cells were followed the eggs were first thoroughly washed in distilled water to free

them as far as possible of adhering electrolytes, as suggested by Loeb (9). In carrying out pH determinations individual eggs were always used and readings were made in triplicate. Between each reading the electrode was washed and checked by readings on standard buffer solutions of known pH values. The electrode vessel was at times put directly into the egg and the fluid drawn up into the capillary, while at other times small punctures into the surface of the egg with fine glass or steel needles were made and the fluid then immediately drawn up into the capillary. No significant differences were found in pH values for fluid drawn up into the capillary by these different methods. In all experiments the time consumed in drawing up the fluid into the capillary and in the taking of pH readings was extremely short, a matter of a few seconds. Eggs were individually taken from solutions by means of pipettes, washed in distilled water in the case of the acid experiments, quickly dried, and excess solution removed from the exterior by placing them on dry filter paper. They were then placed on a small watch-glass, quickly punctured, and pH determinations made on intracellular fluid. In all instances the intracellular fluid alone was used. Twenty to thirty eggs were always used in each experiment with 100 cc. of solution kept in covered finger bowls. Temperature during the entire course of the experiments ranged from 20°–22°C, but for any one experiment did not vary at any time more than $\pm 0.5^\circ\text{C}$.

Observations and Results

Results of typical experiments are shown graphically in Figs. 1 to 7. Unfertilized eggs "stripped" from the female directly into distilled water or sea water give the same internal pH values, showing, as repeatedly pointed out by Loeb (9), that the eggs are little affected internally by distilled water. pH values for eggs from the same individual are fairly constant, while eggs from different individuals exhibit considerable variation—as shown in Fig. 1. These variations in eggs from different individuals are doubtless due to differences in the ages of the eggs obtained at the time of "stripping." The mean average internal pH value for unfertilized eggs at the time of "stripping" is 6.39. Unfertilized eggs kept in distilled water or in sea water and the solutions changed at frequent intervals show fairly constant internal pH values for periods up to approximately 48 hours, after which the values become progressively more acid until the egg finally dies. The most striking difference shown between a fertilized and an unfertilized egg is the marked constancy of internal pH values of the fertilized eggs as compared with the variations in the case of the unfertilized eggs (Figs. 1 and 2). This perhaps is again due to the simi-

larity in condition and age of the fertilized as compared with the various degrees of unripeness, etc., of the unfertilized eggs. Fertilized eggs show an extremely constant pH value, 6.39, even after the developing embryo has increased considerably in size (Fig 2)

It is of some interest to note that the internal reaction of *Fundulus*

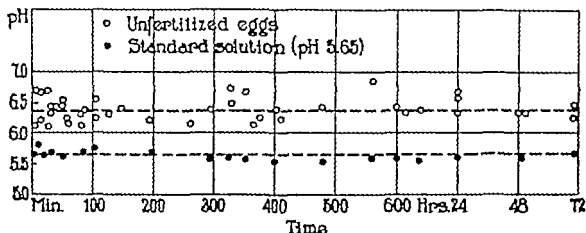


FIG 1 Shows internal pH values for unfertilized *Fundulus* eggs at time of 'stripping' from female and during exposure to sea water for different time intervals. Each point represents average of day in some cases for eggs from same female, in others for eggs from different females.

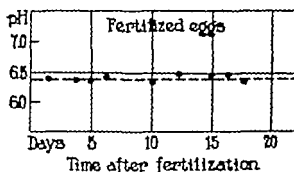


FIG 2 Shows average internal pH values for fertilized *Fundulus* eggs during course of development of embryo. Points represent average values taken from many individual eggs.

egg, a vertebrate egg, is acid in nature despite the fact of its almost constant alkaline sea water environment (pH 8.2). Internal acid reactions, however, have been reported for various forms, protozoa, *Arbacia* eggs, etc. by several authors (1, 3-6)

Inasmuch as Loeb (7, 9) has so strikingly shown the extreme resistance of *Fundulus* eggs to changes in osmotic pressure, to acids,

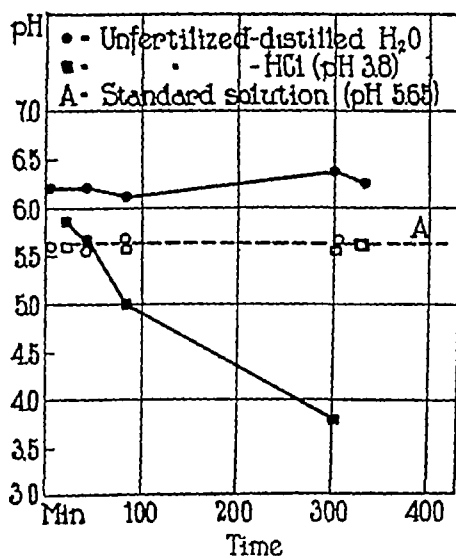


FIG 3 Shows changes in internal pH values of freshly "stripped" unfertilized *Fundulus* eggs exposed to HCl, pH 3.8

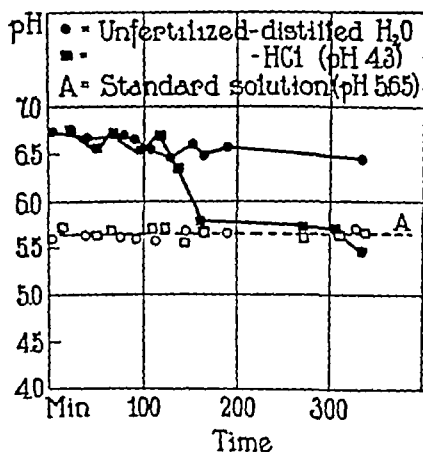


FIG 4 Shows changes in internal pH values of freshly "stripped" unfertilized *Fundulus* eggs exposed to HCl, pH 4.3

etc , and since by the present method the internal pH of the egg could be measured, it was thought desirable to make experiments similar to those of Loeb and to follow internal pH changes as well as func-

tional changes in the embryo Both fertilized and unfertilized eggs were subjected to HCl of different pH values and the rates of internal

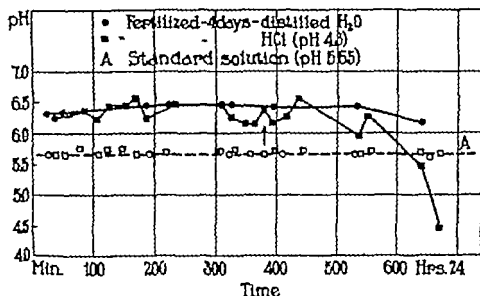


FIG 5 Shows changes in internal pH values of *Fundulus* eggs (4 days after fertilization) exposed to HCl pH 4.3 Arrow indicates time of cessation of heart beat and circulation.

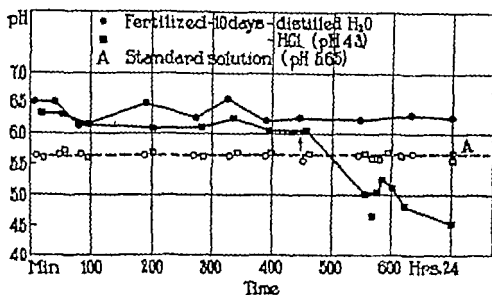


FIG 6 Same as Fig 5 but for eggs 10 days after fertilization Arrow as in Fig 5

pH change were noted Unfertilized eggs seem to be less resistant to HCl than fertilized eggs, as shown in Figs 5 to 7 It is of interest

to note (Figs 3 and 4) that HCl, pH 3.8, enters the unfertilized egg, as judged by internal pH changes, at a rapid and fairly uniform rate. Changes in opacity of the cells closely follow internal pH changes. Less concentrated acid (pH 4.3) penetrates less quickly, as shown in Fig 4. No appreciable changes in internal pH occur for some time after exposure to the acid, as shown by the flatness of the curve (Fig 4) for the first 100 minutes. The fact that the curves show a period during which little penetration of HCl into the egg occurred, followed by a fairly rapid penetration, would seem to indicate a probable surface injury. Fertilized eggs seem even more

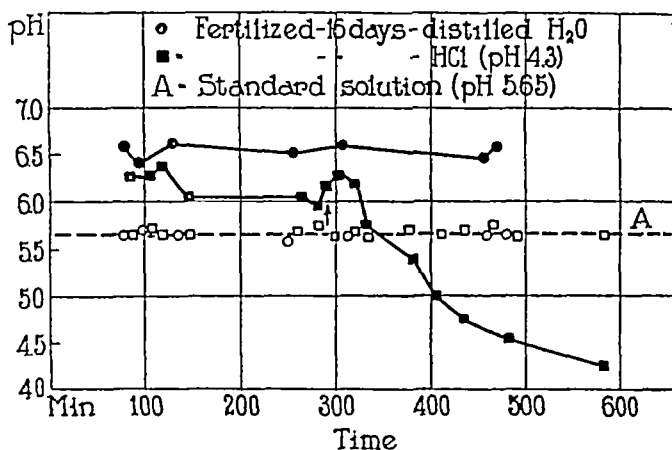


FIG 7 Same as Fig 5 but for eggs 16 days after fertilization. Arrow as in Fig 5

resistant to HCl (pH 4.3) than unfertilized eggs, as shown in Figs 3 to 7. No marked differences in resistance correlated with age of the fertilized eggs seem to exist. When changes in internal pH values begin they usually continue at a uniform and slow rate until the external and internal pH values are in equilibrium (Figs 3 to 7). In all cases at the time when the inner and outer pH values coincided the cell or embryo was dead. Loeb's (7) curves for the rate of penetration of HCl, pH 3.7, into the *Fundulus* egg show during the first few hours of exposure rather a uniform and gradual change in the pH of the external solutions, indicating, according to Loeb, a gradual entrance of the acid into the egg. It seems to the writer that under the conditions

of Loeb's experiments one would hardly be justified in drawing such conclusions.

In studies on the resistance of *Fundulus* eggs to acid, considerable emphasis has been given by Loeb to the assumption that cessation of heart beat or circulation in the developing embryo indicates entrance of the acid through the egg membrane. During the course of the present experiments all eggs were carefully examined under the microscope and the time of cessation of heart beat or circulation in the embryo noted. It will be found by inspection of Figs 5 to 7, on which cessation of heart beat is indicated by an arrow, that considerable variations exist as to a correlation between cessation of heart beat and internal pH changes. As a matter of fact, in several experiments it was found that many embryos with hearts stopped could be made to recover by being returned to sea water. It seems to the author quite possible that surface effects of the HCl might easily account for such functional disturbances without internal changes resulting in the pH of the egg. It is, of course, conceivable that the delicate embryonic heart located so close to the surface of the egg might be affected by the HCl before it diffused further into the egg. Such a conception cannot be checked by the present method since we are dealing primarily with changes in the pH of the egg contents and not with the individual cells of the embryo.

These results are of a preliminary nature and show that by this method we may obtain some idea of the rates of entrance of acids like HCl into the egg of *Fundulus*.

SUMMARY

1 By means of a micro hydrogen electrode and vessel the internal pH values of single egg cells of *Fundulus heteroclitus* have been measured.

2 Unfertilized eggs show a mean average internal pH value of 6.39. Considerable variations in pH values for unfertilized eggs exist and these are perhaps due to variations in the ages of the eggs obtained from different females.

3 Fertilized eggs show a mean average internal pH value of 6.39 with extremely small variations between eggs from different females.

4 No marked differences in pH values for fertilized eggs of different ages were detected

5 Rates of entrance of HCl, as judged by internal pH changes, have been followed for fertilized and unfertilized eggs

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THE PRECISE MEASUREMENT OF HEMOLYSIN

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(Accepted for publication, January 4 1927)

The content in hemolysin of a solution is commonly measured as the amount which is just sufficient to produce complete hemolysis of an arbitrary quantity of red blood cells, usually 0.5 cc of a 5 per cent suspension of washed erythrocytes. The method employed for such measurement gives results which are neither exactly comparable in determinations made at different times, nor highly precise. The susceptibility of erythrocytes to hemolysis is influenced by many factors, and the amount of the minimal hemolyzing quantity must vary accordingly for every specimen of test cells. Lack of precision in the measurement results from the fact that relative differences in hemolysin content between adjacent tubes in a titration series must be great, in order to distinguish the end point, so that the value determined differs often by a large amount from a possible true value.

The method of titration of hemolysin described in this paper was developed for a study of the association of hemolysin with different fractions of immune serum and plasma protein. It measures hemolysin content as the ratio of the hemolytic activity of a given solution to that of standard immune serum. This standard has been usually a portion of the whole serum from which isolated protein fractions have been derived.

This choice of a standard immune serum instead of a given quantity of erythrocytes, as the unit of measurement of hemolysin content, has the advantage that applies to the use of a diphtheria antitoxin for the standardization of toxin and of new antitoxin: the antibody is the most stable biological element of the immune system.

The necessity for a large increment of hemolytic substance in successive tubes in a titration to determine the minimal hemolyzing quantity has long been recognized, and is not peculiar to the immune

hemolytic system With most, if not all hemolytic agents the increment of substance necessary to produce the final 10 or 15 per cent of complete hemolysis of a given quantity of cells is not in proportion to that which brings about the preceding fractional amount of hemolysis, but greatly exceeds its proportion Madsen¹ made this observation first for tetanolysin, and to measure the lytic value of this hemotoxin determined the amount required to produce hemolysis equivalent to that of one-third or one-sixth of the total quantity of red cells used as reagent Schur² used a similar method for the estimation of staphylolysin, and plotted the amounts of lysin against the corresponding amounts of hemoglobin liberated The S-shaped curve so obtained is given also by serum, saponin, and NaOH, according to Handovsky,³ although Miont⁴ had previously reported that the amount of serum hemolysis, with an excess of alexin, is proportional to the amount of sensitizer Brooks⁵ found that the amount of hemolysis is not proportional to the amount of alexin in the presence of a constant amount of sensitizer, but is represented by an S-curve which is similar to those of Handovsky,³ and has devised a method for the titration of complement which makes it possible to compare the amounts of alexin which produce like results in constant time

The method of titration of hemolysin content described here utilizes the same principle, it compares the amounts of unknown and of standard hemolysin which produce a definite fractional amount of hemolysis of a given specimen of erythrocytes when acting in conjunction with a given specimen of alexin

The standard immune serum is freshly diluted for each titration and brought to a concentration such that 1.0 cc will produce almost complete hemolysis of 0.5 cc of a freshly prepared 5 per cent suspension of sheep erythrocytes A series of tubes is prepared containing 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 cc of the standard immune serum dilution A preliminary titration of the unknown serum or fraction is carried out, if its approximate value is not known, and in a second

¹Madsen, Th, *Z Hyg u Infektionskrankh*, 1899, xxxii, 214

²Schur, H, *Beitr chem Physiol u Path*, 1903, iii, 89

³Handovsky, H, *Arch exp Path u Pharmacol*, 1912, lxi, 412

⁴Miont G, *Ann Inst Pasteur*, 1905, vii, 84

⁵Brooks, S C, *J Med Research*, 1920, vi, 399

series of three to five tubes are placed graded amounts of the unknown such that approximately 50 per cent hemolysis of the test cell quantity will be brought about by one of the intermediate tubes in the series. The fluid in all tubes of both series is brought to the same volume, 0.5 cc. of the erythrocyte suspension added with a quantitative or standardized pipette, and alexin representing two units added. For reading in the ordinary Duboscq colorimeter the final volume should be at least 4 cc. Both series of tubes are placed simultaneously into the water bath at 37°C, kept agitated, and withdrawn and placed in cold water as soon as hemolysis is complete or almost complete in the highest concentration of the standard series. After cooling the tubes are centrifugalized and the percentage amount of hemolysis in each tube of standard and unknown series is determined in the colorimeter, using the "highest" tube of the standard series as the 100 per cent standard. This is permissible since complete hemolysis of one "unit" of cells is not the end point chosen in the comparative measurement.

A graph is prepared in which the fractional amount of hemolysis in each tube of the standard series is plotted as ordinate against the corresponding amount of diluted immune serum as abscissa. The curve is S-shaped, it varies slightly in form with each specimen of erythrocytes or alexin. From this curve is obtained the value of the abscissa of the standard corresponding to the fractional amount of hemolysis produced in each tube of the unknown series, or to the 50 per cent ordinate determined by interpolation on a curve drawn through the experimental points of the unknown.

Simple calculation then gives the ratio of the hemolysin concentration of the unknown to that of the standard. The concentration of any solution in units which produce a given fractional amount of hemolysis under the conditions of each experiment may be represented by M/V where M is the dilution, or volume in cc. in which is contained 1 cc. of the serum, or protein fraction referred to the original serum volume, and V is the volume of diluted solution which is required for the given amount of hemolysis. Then the ratio of unknown concentration to concentration of standard is given as follows

$$\frac{M_x}{M_u} \times \frac{V_{ud}}{V_x} = \frac{C_x}{C_{ud}}$$

for each fractional amount of hemolysis

The ratios obtained from varying amounts of hemolysis have been found to agree within 4 per cent if the ordinates chosen lie between 15 and 85 per cent on the scale of ordinates so that the probable error or divergence from the mean is not greater than 2 per cent for any single reading. Consequently a single tube of dilution of unknown may be used for measurement of hemolysin concentration, if its value falls within the limits mentioned.

It is important that both alexin and erythrocytes be fresh and that the cells be washed in only two changes of isotonic suspending medium. More thorough washing of the cells leads to higher values of hemolysis in the first part of the curve, with little effect on the hemolysis of the final portion of the curve, so that greater error is introduced in determining the abscissa of the standard which corresponds to the ordinate of the unknown.

Comparison at different times, with different specimens of erythrocytes and alexin, between the same unknown solution and the same standard has given values that agree within 2 per cent.

SUMMARY

A method is described for the measurement of hemolysin concentration, which makes possible exact comparison of results obtained at different times and with different specimens of erythrocytes and alexin, and gives precise values with an error not greater than 2 per cent.

THE PROTEIN ASSOCIATED WITH HEMOLYSIN IN RABBIT SERUM AND PLASMA.

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(Accepted for publication, January 12 1927)

The immune substances or antibodies do not occur free in the blood, but are found associated with the proteins of the serum or plasma. With certain phenomena in the combination of immune body and antigen, the behavior commonly observed may be a function rather of the associated protein than of the immune body itself. "Protective" or "sensitizing" colloid effects on cell suspensions are brought about by a number of proteins which do not possess immune body.¹⁻⁴ The formation of a film of protein on the surface of the cell has been offered as an explanation of such cases.^{1,2} It is not certain, however, that it is the protein nature of the film in the case of immune sera which is responsible for the changes in the physico-chemical properties of the immune system. On account of the influence of "indifferent" proteins, and of protein derivatives,³⁻⁴ even when present in low concentration, experiments planned to throw light on this problem can be undertaken only with immune body preparations that contain no protein except that intimately associated with immune body. The present investigation was undertaken in the endeavor to obtain such a protein fraction of immune serum or plasma. Hemolysin was chosen as the immune body because of the relative ease and accuracy with which this antibody may be titrated.

¹ Coulter C. B. *J. Gen. Physiol.* 1921-22 iv, 403

² Northrop, J. H., and De Kruif, P. H. *J. Gen. Physiol.* 1921-22 iv, 655

³ Eggerth, A. H., and Bellows, M. *J. Gen. Physiol.*, 1921-22, iv, 669

⁴ Arkwright, J. A., *J. Hyg.* 1914 xiv, 261

⁵ De Kruif, P. H. *J. Gen. Physiol.* 1921-22 iv, 395

⁶ Putter, E., *Z. Immunitätsforsch. Orig.*, 1921 xxxii, 538

Hemolysin, or hemolytic sensitizer, is associated in rabbit serum with the globulin, as is immune body in general,⁷ and has been found chiefly or entirely in the pseudoglobulin fraction⁸⁻¹¹ Recently, however, hemolysin has been described as occurring entirely or in greater part in the euglobulin fraction^{12, 13} The assignment of immune body to one fraction or another must depend on the experimental definition of the fractions of serum

In the present work protein fractions have been obtained from the serum or plasma of rabbits immunized to sheep erythrocytes by dilution with water, and dialysis, after adjustment of the pH to the optimum for separation of the less soluble or globulin fractions The content in hemolysin of the fractions obtained was determined by the method described in a previous paper¹⁴

From serum the fraction commonly known as euglobulin was found to precipitate on dilution at an optimal value of pH 5.9 to 5.8 The amount of protein brought down and the hemolysin recovered in the reprecipitated globulin varied with the degree of dilution the precipitate from a dilution of 1 to 5 contained 1.4 per cent and from 1 to 20 dilution contained in three sera 25, 36, and 45 per cent of the total hemolysin of the corresponding whole serum Further lessening of the electrolyte concentration by dialysis in 1 to 6 dilution of whole serum or of the solution from which globulin had been removed by dilution alone led to the separation of a larger amount of globulin and immune body The pH of the solutions was adjusted to 5.9, dialysis was carried out in collodion sacs for 1 to 7 days The total amount of immune body recovered by this procedure represented 45, 28, and 62 per cent of the total present in three sera The first value represents an increase of 9.5 per cent in terms of the con-

⁷ Pick, E. P., *Beitr. chem. Physiol. u. Path.*, 1902, 1, 351

⁸ Fuhrmann, F., *Beitr. chem. Physiol. u. Path.*, 1903, III, 417

⁹ Meyer, K., *Arch. Hyg.*, 1908, LXXII, 114

¹⁰ Ruppel, W. G., Ornstein, O., Carl, J., and Lasch, G., *Z. Hyg. u. Infektionskrankh.*, 1923, XCIV, 188

¹¹ Locke, A., and Hirsch, E. F., *J. Inf. Dis.*, 1924, XXXV, 519

¹² Otto and Sukenikowa, *Z. Hyg. u. Infektionskrankh.*, 1924, CI, 398

¹³ Laubenheimer, K., and Vollmar, H., *Z. Hyg. u. Infektionskrankh.*, 1926, CVI, 202

¹⁴ Coulter, C. B., *J. Gen. Physiol.*, 1926-27, X, 541

tent of the whole serum over that obtained by dilution to 1 to 20 alone, at pH 5.8

This globulin precipitated most promptly from aqueous solution at pH 5.65. No fractionation of the protein was possible by variation of the pH of aqueous solutions. If, however, NaCl in substance was added to the concentration of isotonicity to an aqueous solution of the globulin, a precipitate was obtained which varied in amount and in time of appearance in different specimens and formed in a rather wide range of pH with an optimum near pH 5.0. It was often incompletely soluble in water at pH 7.0 to 8.0.

Preparations from two sera had a hemolysin content of 0.15 and 0.63 per cent of that of the corresponding whole serum. The hemolytic activity of the globulin solution after the separation of the salt insoluble precipitate was found to be 1, 5, and 12 per cent greater than before its removal. The salt insoluble fraction appears thus to act as antialexin, this property is destroyed by heating to 50°C. The nature of this fraction is uncertain. It was at first regarded as a denaturation product, as described by Wu and Yen,¹⁴ as a result of the hydron concentrations to which the serum had been subjected. Later work with plasma suggested that it may be residual fibrinogen.

The hemolysin content of the solution which remained after dialysis was similar to that obtained with plasma, which is described below.

In obtaining plasma from immunized rabbits, coagulation was prevented by sodium citrate or potassium oxalate. On dilution of plasma with water, a flocculent precipitate consisting mainly of fibrinogen appeared at the optimal reaction of pH 6.4 to 6.1.

This fraction from a 1 to 5 dilution contained 2.3 and 2.5 per cent, and from 1 to 10 dilution 2.6, 5.5, and 6.5 per cent of the hemolysin of the corresponding whole plasma. In carrying out the titrations of solutions containing fibrinogen it was necessary to make the initial dilutions with water, to prevent fibrin formation. The fibrinogen itself in this fraction appears not to carry immune body: the hemolytic titre after the separation of fibrin by initial dilution in saline solution was the same within the limit of error in measurement, as

¹⁴ Wu, H. and Yen, D. *J. Biochem.*, 1924-25, iv, 345.

when clotting was prevented by initial dilution in water. Furthermore, the presence of fibrinogen under certain conditions depresses the apparent hemolytic activity of the immune protein, as found and described below, with the second fraction of plasma.

Globulin and a second portion of fibrinogen were separated from the remaining plasma solution by dialysis at pH 6.1 against distilled water. Thymol was added, and dialysis carried out at 5°–10°C for 3 to 15 days. The greater portion of water-insoluble protein flocculated in 48 hours, a further small amount, which was identical in its solubility with the first portion, separated slowly during 15 days or more. The precipitate, which appeared on dialysis, was soluble in water, and showed an optimum for flocculation from aqueous solution at pH 6.1. An hemolysin content of 33, 48.5, 48, 62, and 68 per cent of that of the whole plasma was obtained in this fraction. Longer dialysis was employed with the later experiments, with resulting increase in separation of hemolysin as indicated by the values which are given in the order in which the experiments were carried out.

The fibrinogen present in this fraction separated out as fibrin when NaCl was added to isotonicity to an aqueous solution. Fibrin formation took place at reactions between pH 8.0 and 5.6, with an optimum about pH 7.2, separation was slow and three or more clottings were observed if each clot was removed as it formed. Within the limits of pH given, the formation of fibrin gel was most rapid and complete within certain concentrations of NaCl or CaCl_2 , the limits were not precisely determined, but approximated isotonicity for NaCl. On the acid side of pH 6.0 fibrinogen separated out as a granular precipitate which was greatest in amount at pH 5.0 to 4.8. Fractions of immune protein which contained fibrinogen showed the greatest hemolytic activity, when brought in saline solution to pH between 8.0 and 4.0, at the reactions optimal for formation of fibrin gel. The presence of fibrinogen, possibly because of the form in which it existed, in solutions at other reactions appears to depress the hemolytic activity.

The total N associated with one hemolytic unit, of the usual value, was found with globulin preparations freed from fibrinogen to be 0.00038, 0.00034, and 0.00007 mg. with three different specimens.

The values are very close to those of Locke and Hirsch¹⁴ for hemolysin obtained by dissociation from specific combination.

The hemolysin present in the plasma solution from which water insoluble globulin had been separated by dialysis varied inversely with the amount recovered in the globulin fraction, a minimum of 2.8 per cent of the total was found in a specimen which had been dialyzed for 15 days.

DISCUSSION

The globulin with which hemolysin is associated appears to exist in plasma as an adsorption complex with fibrinogen, the latter determines the optimal pH of flocculation from aqueous solution of the complex. The conditions under which fibrinogen forms a fibrin clot, or gel, recall those found by Falk¹⁴ for gel formation by banana protein, in both cases gel forms only on the alkaline side of pH 6.0 and within certain limits of salt concentration. The relation of Ca to fibrin formation in the solutions studied is not as evident as in the case of banana protein, Ca is known to be necessary for clotting only in the first step, for which blood Ca was available in these plasma preparations.

The granular form in which fibrinogen separates from isotonic NaCl solution at pH 5.0 to 4.8 suggests that a similar precipitate from serum protein solutions is residual fibrinogen. Both fibrinogen and the salt-insoluble protein from serum carry down no hemolysin or only an insignificant amount which may represent adsorbed globulin. The depression of hemolytic activity by amorphous precipitate of fibrinogen is perhaps due to adsorption of alexin.

Extraction of immune globulin preparations, with 8 to 20 volumes of 95 per cent alcohol at 0°C, caused a loss of at least 90 per cent of the immune body. The evaporated residue of the alcoholic extract contained both protein and lipid, which neither alone nor together showed hemolytic activity. At the same time, the hemagglutinating property of the immune protein was found considerably intensified after alcoholic extraction. The partial denaturation of the protein by alcohol destroys the hemolytic activity, which must depend upon a different property of the protein from its agglutinating action.

¹⁴ McGuire G. and Falk K. G. *J. Gen. Physiol.*, 1921-22 iv 437

The water-insoluble globulin which was obtained by dialysis was found to contain both euglobulin and pseudoglobulin, defined as fractions precipitated by one-third and one-half saturation with ammonium sulfate. Although the separation of pseudoglobulin and immune body from serum or plasma by dialysis at optimal pH was not complete, the present work confirms the view of Pauli and Adolf¹⁷⁻¹⁹ that no distinction can be made between a water-soluble and a water-insoluble globulin.

SUMMARY

1 The water-insoluble globulin with which hemolysin is associated, may be separated from immune serum or plasma by dilution and simple dialysis at optimal pH.

2 This optimum in plasma is influenced by the presence of the fibrinogen.

3 Fibrinogen carries no immune body, or only an insignificant amount, when present in immune body solutions in other form than fibrin gel, it depresses the hemolytic activity. The conditions for the formation of fibrin gel are similar to those for the formation of a gel by banana protein.

4 The hemolytic activity is a more labile property of the immune protein than the agglutinating activity, hemolysin is destroyed, hemagglutinin shows an apparent increase, as a result of alcohol extraction.

¹⁷ Pauli, W, *Biochem Z*, 1924, clu, 355

¹⁸ Adolf, M, and Pauli, W, *Biochem Z*, 1924, clu, 360

¹⁹ Adolf, M, *Klin Woch*, 1924, III, 1214

"GALVANOTROPISM" OF ROOTS

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(Accepted for publication January 20, 1927)

I.

The galvanotropic orientation of animals may probably be used for analysis of certain features of central nervous activity¹. Therefore it is necessary to obtain a conception of the mode of stimulation by passage of direct current. In relation especially to the results of studies, in this laboratory² and elsewhere, upon the conductance of plant cells, and because of the structurally simpler conditions of response, we have paid attention to the well known "galvanotropic curvature" of roots.

Every one interested in plant irritability has always considered the "galvanotropic response" of the roots as one of the most remarkable facts described. Discovered by Elfving in 1882, the "galvanotropic response" was studied by Müller Hettlingen, 1883, Brunchorst, 1884, Ruschaw, 1885, Ewart and Bayliss 1906, Schellenberg, 1906, and in a rather long paper by Gassner, 1906. Indications are given also by Szűcs, 1913, and a general review of the subject can be found in Stern's book (1924).

Based on all these observations, it is generally admitted that for high densities of current, or for long exposures, a curvature towards the + pole is obtained (so called Elfving's curvature), that, on the other hand, for lower densities of current, or for shorter exposures,

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¹ Cf. Crozier, W. J. 1926 *J. Gen. Physiol.* ix, 395

² Osterhout, W. J. V. 1926 *J. Gen. Physiol.*, viii, 131. Blinks, L. R. 1926 The permeability and electrical conductivity of single cells Thesis Harvard University Cambridge, 1926

the curvature is directed toward the — pole (genuine "galvanotropic response") This last is considered as a tropism, the first curvature being said to be purely traumatic

The definition of the true "galvanotropism" of the root can be therefore expressed in the following manner It is a growth curvature, directed towards the cathode, located in the region of maximum growth, irreversible by plasmolysis and requiring the presence of the tip of the root

The origin of this curvature is naturally the real question For the explanation of this origin several theories have been advanced Brunchorst sees in the curvature an injury by the electrolysis products, especially the H_2O_2 that may be produced Rischaw considers the phenomenon similar to the electrosmotic water displacement in the albumin cylinders of du Bois Reymond's experiments Ewart and Bayliss attribute the response to chemotropic stimulation by the products of electrolysis, all idea of traumatic curvature being excluded

On the contrary, for Gassner the facts may be explained as a traumatic response of the organism to a unilateral injury of the tip of the root

In fact, to all these explanations the same objection may be made the technical conditions were often too crude and, in a certain number of cases, too vague to be used adequately as bases for explanations For instance, Ewart and Bayliss used platinum electrodes directly in contact with the plant In other cases, it is true, so called unpolarizable electrodes were employed Others (Brunchorst, Gassner, Schellenberg) used carbon electrodes, often dipping directly in the same liquid as the root tips but sometimes surrounded by a septum made by a porous plate For many cases the density of current corresponding to the position of the roots in the trough is not known

It was thought interesting for these reasons to reproduce these experiments, trying always to avoid the errors pointed out

The actual experiments were carried out keeping in mind the following points (1) Reduction of polarization products by use of unpolarizable electrodes, (2) prevention of diffusion of the products of electrolysis by use of agar blocks, (3) gradient of densities of current easily controllable by use of troughs with definite geometrical shapes and sizes

II

Technique

1 Trough 1—Paraffine blocks were carved out following the indications of Fig. 1. These troughs are characterized by their variable cross-section, one end being a square of 5 cm. of side, the other end being 2 cm. \times 5 cm. The distance of these two sections is 25 cm. and each section is closed by a block of agar gel. The agar was purified agar which had been soaked in 2 per cent HCl for 24 hours, then in 1 per cent ammonia for 12 hours, then subjected to running water for 48 hours, all with frequent shaking. After this treatment the water was more or less pressed out and the agar washed several times with distilled water, this being also used to make the gel, of which the concentration was 10 per cent in dry weight of agar. This agar gel is

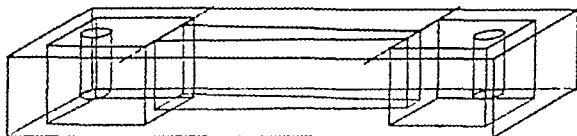


FIG. 1. The figure represents Trough 1. In Trough 2 the blocks of agar are omitted and replaced by porous plates in the vertical planes of the dot-dashed lines.

poured in place, care being taken to obtain a plane surface at both ends of the trough (the real electrode surfaces) and cups being provided in each block for the electrodes. These cups as well as the trough are filled to a definite height with tap water or with a balanced salt solution (diluted Knop solution). This liquid was removed immediately after each experiment and the trough was washed for several hours by a continual flow of tap water.

2 Trough 2—The other type of trough is similar, the differences being only in the replacement of the agar blocks by porous plates 2 mm. thick.

3 Electrodes—The electrodes used were carbon cylinders 2.5 cm. in diameter, a good contact being assured for the leads, or unpolarizable electrodes, either calomel electrodes (employed in a few cases only,

the inner resistance being too high) or of zinc-zinc sulfate. In this last case the electrodes, which were kept in a concentrate solution of zinc sulfate, were rapidly washed before use under running tap water. Furthermore, care was taken to have the same hydrostatic level in cups and electrode.

4 *Current*—The potential differences applied to the electrodes varied from 0.5 volts to 115 volts. The density of current (according to the position of the root in the trough) varied from 0.058 ma./sq. cm. to 1 ma./sq. cm. The time of exposure to the action of the current was changed between 15 minutes and 360 minutes.

5 *Material*—The plants used were *Vicia faba* (broad Windsor beans) and *Phaseolus vulgaris* (Burpee's improved bush lima beans). The seedlings were grown in sawdust at 20°C and their roots were practically straight. They were used when the length of the roots was 4 to 6 cm. In a few cases experiments were made with secondary roots with the same results as with primary ones. Normally the roots dipped for 8 to 10 mm. in the solution.

III

Results

1 When Trough 1 is used alone, whatever the P.D. or density of current may be, and whatever the duration of the experiment (between 15 minutes and 360 minutes) no curvature ever occurs.

When Trough 2 is used, with the same conditions, curvatures are shown if carbon electrodes are used and always towards the cathode.

A test was to put Troughs 1 and 2 in series, as the objection could be made that the density or time was deficient. Under these conditions curvature appears in No. 2, no curvature in No. 1, so the current and time of exposure were large enough to produce "galvanotropic" responses.

The immediate conclusion to be drawn from these experiments is that "galvanotropic" curvature is produced by the products of electrolysis.

2 Was therefore the galvanic current necessary? Sets of seedlings were put with the root dipping for 1 cm. in the water of Trough 2, for 24 hours, after the current had been passed through the water for

2 and 4 hours. Care was taken not to move or disturb the water during or after the electrolysis

The immediate result of this test was to show a slight but definite curvature—in fact less than when the current was acting directly on the seedlings and practically not directed toward one pole or the other. The angle of deflection of the tip of the root was about 15° to 20°

If the same experiment was made, with the same conditions (carbon electrodes, 2 or 4 hours previous passage of current) with Trough 1, no curvature is shown

This fact proves that the blocks of agar, 10 cm. in length, are sufficient to prevent the diffusion of products of electrolysis or to slow it up so that they do not reach the middle part of the trough in time to affect the seeds

There is therefore a difference in the curvatures when current is present or absent but the difference is merely quantitative

We must for this reason recognize in the "galvanotropic" curvature a double effect—the first being produced by the electrolysis products, the second being the further increase of the first under the persistence of the electrical current.

The second test, showing the influence of the electrolysis products is, in fact, sufficient to show that the primary effect on the root is a traumatic one.

3 Another way to show this was to injure the root before the experiment and to place it then in Trough 1. If really injury is the first step, curvature must occur under these conditions, with non polarizable electrodes. And it does

Roots were placed for 2 to 3 minutes in a solution of copper nitrate N/100, then in Trough 1 filled with tap water. The current was passed for 60 minutes. The plants were left in place in the same liquid for 24 hours (as in all the other experiments) after the passage of the current. At the end of this time a definite cathodic curvature was shown

Experiments made after immersion of the roots for 10 minutes in the same copper solution did not give curvature

Microchemically it is easy to show that during the short exposure the two external layers of cells are permeated by the copper ion, and

that in the second case a much larger number of layers are injured. The latter injury corresponds practically to the killing of the root.

So, to produce the "galvanotropic" curvature in the absence of electrolysis products, the injury must be definite but not too large. A certain amount of tissue must remain in the root to react. The curvature is in fact a response from injured but not dead tissues.

IV

Interpretation

How may we try to explain these facts? As was pointed out previously the response must surely be developed in two steps (or more).

Let us consider a root dipping in the water between the two electrodes. This body in the electrical field naturally repels the lines of flow around itself, as we know that the living cells are practically non-conducting for direct current. But under these conditions there may be accumulation on the opposite sides of ions of opposite signs. And this may be sufficient to injure the epidermic layer of the root. What are the ions which are so toxic? It may be said that perhaps any ion present in the solution used will act in this way.

In a root of circular cross-section we may thus consider two opposite halves in the epidermic layer, both injured and probably with a number of the cells killed, and acting for the remaining part as two electrodes directly applied on the internal tissues. These electrodes determine, inside of the root, an electrical field, the conducting paths being made by the cellulosic membranes imbibed with aqueous solutions. The resulting electrolysis acts now on more deeply situated cells.

The produced ions are formed in such loci that they may act directly on the plasmatic surfaces, inducing in these such changes that the relative dielectrical resistance diminishes and that free ions may migrate in or out of each cell. The result is necessarily that under the directing action of the electrical field, in all cells through the root, chains of + and - charges are formed, each cell having a + charge on the cathodic side and a - charge on the anodic side. The perduration of the current continues the same action in the same way and causes finally a relative increase of anions on the anodic side and of

cations on the cathodic side. The two halves of the root are brought, by this process, to be ionically different. The early effect of the anions on the anodic side seems not to prevent growth. The relative accumulation of cations, on the contrary, slows the growth on the cathodic side, and by further increase stops it completely. The effect of this differential state is a bending towards the cathode.

V

In the earlier papers on galvanotropism another type of curvature has been described, the so called Elfving's or anodic curvature. With the device described in the present paper it has always been impossible to obtain this effect, even when the root was deeply dipping in the water. We may draw from this fact the deduction that in the cases described, with very high densities of current, other factors were interfering.

The same can be said as to the *S*-shaped curvature described by Gassner when more than the tip of the root dips into the liquid: in the present conditions of experimentation no case of this bending was found. Is it therefore to be deducted that this type of curvature does not exist? No, probably; but that under the conditions of experimentation, insufficiently described in the papers to which reference is made, other effects are occurring. Other experiments are necessary to ascertain the conditions of production of these curvatures, as well as the change in conductance of the tissues showing or not showing these reported curvatures.

SUMMARY

1 New experiments, made in such a way to eliminate as completely as possible products of polarization and the migration of such products when formed, have shown that the exhibition of galvanotropic curvature in roots is mainly dependent upon such products, since no curvature appears when they are excluded.

2 The polarization products injure the external layer of cells of the root: this allows these cells to act as electrodes directly applied on the internal tissues. The inner electrolysis produces such changes in the interior cells that they may be considered as becoming ionically different. This differential state is responsible for curvature.

3 "Galvanotropism" of roots, therefore, cannot be regarded as exactly comparable to the galvanotropic orientations of certain animals, but is essentially dependent upon injury

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THE FREE ENERGY OF NITROGEN FIXATION BY LIVING FORMS

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The view point that energy is required for the fixation of nitrogen by the various forms of life seems to be generally taken for granted among biologists

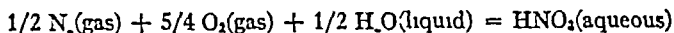
It is the purpose of this paper to present evidence of a simple thermodynamic nature against the soundness of this current conception. It will maintain that, contrary to the current view, energy may not be required for fixation, and that moreover, in fixation considerable energy may be released and placed at the disposal of the organism. In contending for a position so plainly the reverse of the old, important thermal data will be used which has been obtained only in the last decade.

This paper, as so many others, is indebted to Lewis and Randall¹ for most of the free energy values and conventions to be used. Unless it is otherwise stated, the temperature of all the reactions cited is 25°C. The heat of reaction and the free energy of reaction will be negative when heat and free energy respectively are liberated.

Nitrogen fixation may be defined as the primary step or steps in the formation of a simple nitrogen compound such as ammonia or nitrate from pure elementary nitrogen. It is to be distinguished from later changes in which ammonia and nitrate are formed one from the other, and also from the building up of higher compounds with carbon. When an organism once forms a simple compound from elementary nitrogen, the energy relations are those which would exist were it to be supplied with such a simple compound and we must discriminate between the energy of nitrogen fixation and that of nitrogen metabolism.

¹ Lewis, G. N. and Randall, M. *Thermodynamics*, New York: 1st edition 1923

The standard free energy, ΔF° , of the reaction



is a small positive value, showing that at standard conditions this reaction is not spontaneous and will proceed only with the addition of free energy. At standard conditions all the reacting substances have unit activity, that is to say, the gases have fugacities of one atmosphere (for the present calculations their pressures may be considered equal to their fugacities), the water is pure, liquid, and at atmospheric pressure, and the concentration of the nitric acid is at the hypothetical 1 molal. Let us now calculate the conditions in the plant when the above reaction will proceed without the addition of free energy, that is to say, when the free energy, ΔF , will be negative. Avoiding unwieldy overrefinements, let us consider as appropriate approximations, the pressure of nitrogen, 8 atmosphere, the pressure of oxygen, 2 atmosphere, the plant sap to have the same activity as pure water, and the activity of the nitric acid formed to be unaffected by the various foreign substances in the sap.

The standard molal free energies of nitrogen, oxygen, water, and nitric acid are respectively 0, 0, $-56,560$, and $-26,500$ (calories). Hence in this reaction ΔF° is 1780 . Now,

$$\begin{aligned} \Delta F^\circ &= -RT \ln K = -1364.9 \log K \\ \log K &= \log (\text{HNO}_3(\text{aq}) / (\text{N}_2)^{1/2} (\text{O}_2)^{5/4}) = -1780/1364.9 = -1.304 \quad \text{Or, } K = 0.0497 \\ \text{activity HNO}_3 &= K \times (8)^{1/2} \times (2)^{5/4} = 0.067 \end{aligned}$$

From the table containing the activities of nitric acid at various concentrations this value corresponds to a concentration of just 1 M. This means that, assuming the plant can catalyze the reaction, nitrate will form in the plant with the liberation of free energy so long as its concentration remains below 1 M or 6200 parts per million by weight. At this point ΔF no longer has a negative sign but is equal to zero, since this is the equilibrium concentration.

The heat of the reaction is $-49,100 - (-68,310/2) = -14,940$. Owing to the fact that the plant sap rarely if ever reaches a concentration of 6200 ppm, for every mol of nitrate which forms from air and water according to the above equation there are 14,940 calories of heat (not work) liberated for partial use as chemical energy. The

more dilute the concentration at which the nitrate is formed the greater is the amount of available work (not heat) placed at the plant's disposal. Since nitrate in the plant sap is removed soon after its appearance, its concentration probably never rises much above, let us say, 001 μ . If we assume a maximum concentration (i.e. stationary state) of nitrate in the plant sap of 6 ppm or 0001 μ , and that the activity coefficient at this concentration is unity, then the following calculations show that the free energy is -7870 per mol of nitrate formed

$$K = (.0001)^2 / (.8)^2 (.2)^2 = 8.6 \times 10^{-4}$$

$$\Delta F = \Delta F^\circ + RT \ln K = 1780 + 1364.9 (-7.07) = -7870$$

Similar calculations show that after making the permissible assumption that the heat of the reaction would not change significantly under conditions of such extreme dilution, the free energy of the reaction would equal the heat of the reaction, -14,940, that is to say, the process would be 100 per cent efficient, when the concentration of nitrate was $7 \times 10^{-4} \mu$ or 4 ppm. In the sap of some plants this is the highest concentration to be found. Indeed, in studies by Hoagland and Davis with the sap of *Nitella* cells it was found that, although the pond water in which *Nitella* was growing contained about .5 ppm of nitrate, the cell sap contained no detectable amount. If this small concentration of nitrate results from the rapid use made of nitrate by the cell, it is evidence of how efficiently the fixation of nitrogen may be accomplished. Comparison of the free energy (-7870) at a nitrate concentration of 6 ppm with the free energy (-14,940) at the lower concentration of only 4 ppm shows how quickly the free energy increases as the concentration of nitrate decreases.

A citation from Lewis and Randall² will illustrate the significance of this reaction. "It is to be hoped that nature will not discover a catalyst for this reaction, which would permit all of the oxygen and part of the nitrogen of the air to turn the oceans into dilute nitric acid."

Although foreign to the discussion of nitrogen fixation, it might be remarked that the energy needed for the reduction of nitrates following their fixation may be supplied by radiant energy in the case

² Lewis and Randall, p. 568.

of higher plants In leguminous plants, should the nitrogen-fixing bacteria produce nitrates, they might release these into the sap where they could be carried to the leaves and reduced by sunlight at no energy expense to the organism

It will be noticed in the foregoing equation that oxygen gas is required as a reactant Does the equation for this reason fail to serve for anaerobic organisms, such as *Clostridium*, which can fix nitrogen in the absence of oxygen? We may escape this conclusion by postulating the formation of oxygen gas incidentally to some other metabolic process, a feasible supposition in absence of data known to preclude a small but continuous production of this gas during fixation A continuous and restricted production of the gas may occur and yet escape detection, owing to the immediate use made of it by the organism The fact that, if such a source be supposed, the activity of the oxygen in the equilibrium constant would be reduced, would affect the conclusions at issue only as regards quantities involved

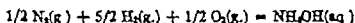
Against the value of this possible source of oxygen as support for the main contention of this paper, it may be objected that, assuming the production of oxygen gas under anaerobic conditions, would not energy be required for its production, and so leave on hand the same occasion as before for assuming an energy requirement for the fixation of nitrogen by anaerobes?

Admittedly, an examination of the score of available free energy data of organic compounds conclusively suggests energy requirement for the production of oxygen A striking and obvious case is found in the reaction of formic acid to yield oxygen and formaldehyde, the standard free energy having a positive value of about 60,000 calories, an enormous amount when considered relatively to the free energies of either of the compounds, which are, respectively, $-87,920$ and $-30,000$ (approximately) And in general this behavior might be expected from most if not all organic compounds However, there might be exceptions, since such inorganic exceptions can be found In the reaction of HBrO_3 to yield HBrO and O_2 , 14,280 calories of heat are liberated and the standard free energy amounts to $-21,980$, and there are several inorganic reactions similar to this one, which is given merely as an example of possibility but not with a view to application Hydrogen peroxide is another example and the yield

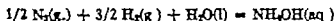
of free energy is even larger than in the case just cited. There might be organic peroxides with a similar behavior.

However, in anaerobic processes oxygen need not be considered. The production of aqueous ammonia from nitrogen and hydrogen is attended by a negative standard free energy change of 6300 calories and a negative heat of reaction of 20,300 calories. This exothermic and free energy yielding production of ammonia resembles that of nitric acid, and a similar correspondence holds between the effects which result from the formation and removal at high dilution of the two compounds ammonia and nitric acid.

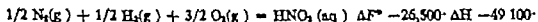
Out of curiosity let us glance at the standard free energy which would be yielded if oxygen were present also. If the reaction proceeded according to



rather than in the manner above,



the standard free energy would be -62,860 as against the former -6300. This is a great increase, owing for the most part to the fact that oxygen of water as a reactant is replaced by oxygen gas. In the same way in the aerobic fixation of nitric acid if hydrogen were present making the reaction proceed

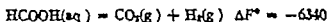


then a great deal more free energy would be evolved than in the original case discussed.

In these reactions the need for hydrogen production might be satisfied from several independent sources with no expense of free energy by the organism.

In anaerobic processes hydrogen is often an end product incidental to metabolism, and would waste if not conserved in fixation.

In both aerobic and anaerobic metabolism many reactions, more particularly the breakdown of organic acids, may proceed spontaneously to yield both hydrogen and energy. For example,



It may be argued that the organism sustains a net potential if not actual loss of energy when the reaction proceeds in this manner, rather than by combustion, when 61,000 calories of heat are yielded, whereas in the reaction cited the heat of the reaction is approximately zero. To this objection it is enough to answer that if no oxygen were present this energy could not be yielded. But assuming an occurrence of oxygen, then since the heat of combustion is less by some 40,000 calories than the heat of formation ($-101,000$) there results a veritable gain of energy upon replacement of the HCOOH molecule. To object that the HCOOH molecule is not replaced under certain conditions is unwittingly to concede that under these conditions HCOOH is a waste product. Were HCOOH derived from glucose and oxygen gas, instead of from its elements, the difference between the heats of formation of $\frac{1}{6}$ mol of glucose and 1 mol of HCOOH , that is to say, the veritable gain of energy, is still larger than the value 40,000 calories by 10,000 calories. And if the glucose were derived from carbon dioxide and water, instead of from its elements, then an enormous amount of energy is required for fixation providing that (1) it is not fixed as HNO_3 , (2) it is fixed as ammonia, the hydrogen coming solely from the glucose (or ultimately from the water), (3) the intermediate product is not a waste product. In terms of standard free energy, 65,900 calories are required at a pH of about 5, increasing as the sap becomes more alkaline, to a value of 78,500. The free energies would differ relatively little from the standard free energies (approximately 10 per cent). Such a reaction will be referred to later in discussing a paper by Linhart.

There are several organic acids whose heats of formation are greater than their heats of combustion, even greater than in the case of HCOOH . These acids are often the end and therefore waste products of anaerobic metabolism, not to say of aerobic metabolism. Indeed, Stoklasa³ reports finding formic, acetic, butyric, and lactic acids in pure cultures of *Azotobacter*, an aerobic form. In these same aerobic cultures hydrogen gas was evolved.

Surprising as it may perhaps seem, an additional source of hydrogen

³ Stoklasa, J., *Centr. Bakt.*, 2 Abt., 1908, xxi, 506

gas is the atmosphere. According to Spoehr,⁴ the atmosphere contains 01 per cent by volume of hydrogen. That this estimate is meritorious, and in addition one which varies little, is indicated by a summary of the experimental determinations given by Mellor.⁵ The estimates of various investigators range from 019 per cent (Gautier) to 003 per cent (Rayleigh), the majority falling near the mean of these two values. In the following discussion the value of 01 per cent will be used. This particular value can hardly mislead since the probable variation from it has been indicated.

This concentration of hydrogen, instead of being small, is about equal in volume to the carbon as carbon dioxide in the atmosphere. By weight the ratio of hydrogen to carbon is 1 to 6. The corresponding ratio by weight in either proteins, carbohydrates, or fats is from 1 to 6 to 1 to 8. In bacteria⁶ it is about 1 to 8. The quantities by weight of carbon and hydrogen in the atmosphere can therefore be considered equal when judged by the needs of the plant.

The following calculations will call attention, with the great suggestiveness of free energy calculations, to the potential importance of atmospheric hydrogen for plant metabolism, and in particular, for nitrogen fixation.

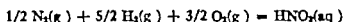
The equilibrium concentration of ammonia which might form from the nitrogen and hydrogen of the atmosphere, with the assistance of catalysts, is .2 ppm in alkaline sap.

$$K = -6340 / -1364.9 = 4.65$$

$$\log K = \log (\text{NH}_4\text{OH}(\text{aq})) / (\text{N}_2)^{\frac{1}{2}} (\text{H}_2)^{\frac{3}{2}} (\text{H}_2\text{O})$$

$$\text{activity NH}_4\text{OH}(\text{aq}) = K \times (.8)^{\frac{1}{2}} (.0001)^{\frac{3}{2}} = 4 \times 10^{-6} M = 2 \text{ ppm.}$$

This value of 2 ppm corresponds to the equilibrium concentration of 1 M for nitric acid. When hydrogen is also used to form nitric acid according to the equation



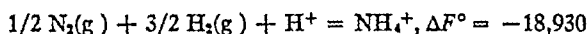
⁴ Spoehr H. A. *Photosynthesis*. American Chemical Society Monograph Series New York 1926 36

⁵ Mellor J. W. *Comprehensive treatise on inorganic and theoretical chemistry* London, 1922 1

⁶ Russell E. J. *The microorganisms of the soil* London 1923 39

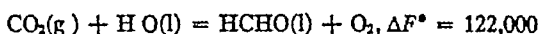
the equilibrium concentration of nitrate is 16 M or 10,100 ppm, an increase of 60 per cent

The equilibrium concentration of bases will be raised by a greater hydrogen ion concentration while that of acids will be raised by a lesser hydrogen ion concentration. Thus in the equation

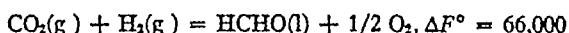


if the sap is assumed to have a pH of about 5, the equilibrium concentration is raised to 6 ppm, a threefold increase over that in alkaline sap

In passing, it will reward us to suggest how this large amount of hydrogen in normal air might play a rôle in the carbon dioxide assimilation of aerobic forms. For instance, if the hypothetical assimilation reaction



were to proceed



the standard free energy required for the fixation of carbon dioxide would be halved. In this case it is important to determine the actual free energies, since the activity of the hydrogen gas is but 1/10,000 that of water. At a concentration of HCHO of 1 part per million, calculations show that there exists a negligible difference (2 per cent and 5 per cent) between the free energies and the standard free energies. For two reasons this might have been anticipated, the standard free energy values are very large, with the exception of oxygen all the substances occur to their first powers in the activity coefficient.

While we need hardly resort to the last named equation to explain carbon dioxide assimilation by higher green plants, in view of the overwhelming evidence to the contrary which has accumulated since the time of Joseph Priestley, it may well be that those organisms, such as the sulfur bacteria, denitrifiers, hydrogen fixers,⁷ elementary carbon fixers,⁸ and others, which reduce inorganic forms of carbon

⁷ Kaserer, H., *Centr. Bakt.*, 2. Abt., 1906, xvi, 681

⁸ Potter, M. C., *Proc. Roy. Soc. London, Series B*, 1908, lxxx, 239

without the aid of radiant energy, derive their energy not solely by the oxidation of non-carbonaceous substances with oxygen gas, but partially, at least, by the reduction of CO_2 or other substances with hydrogen gas. In the same speculative vein it may be suggested that there is some degree of identity between those organisms which can fix nitrogen and those organisms which can utilize hydrogen.

It has now been shown that nitrogen can be fixed both exothermally and with the yielding of free energy either as ammonia or nitrate in aerobic processes and at least as ammonia in anaerobic processes.

Some may think that in nitrogen fixing organisms the simple compounds like ammonia, nitrate, and others are never produced directly, and that what occurs is always the immediate formation of higher compounds with carbon. In other words, the plant may never be able to take proper advantage of the energy yielded by simple fixation. If so, and if energy can be plainly shown to be necessary for the arrangement of nitrogen into higher compounds, then there would be great reason to accept the view now held so widely, that energy is essential for the fixation of nitrogen.

To this argument which on the surface appears to carry weight, there are several objections. The author feels it advisable to enter rather fully into these.

First, physiological chemistry supplies no evidence which makes it probable or improbable.

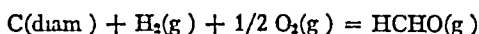
Second, the formation of complex compounds from the simpler takes place very generally with the liberation of energy.

Third, to suppose that complex nitrogen compounds like amino acids can be formed in no more than one step would be contrary to the present principles of organic chemistry. To say the least, a very high (if not unheard of) order of reaction would need to be supposed to bring the direct formation of these compounds in keeping with our present information.

Fourth, leaving aside the question of an energy requirement in the formation of higher compounds from nitrogen gas, it is nearly certain that energy is not essential for their formation from ammonia. Appearance in this instance must not pass for fact. The energy needed to form the higher compounds may be the sum of endothermic changes of the carbon molecules and exothermic changes of the nitro-

gen molecules, to use a distinction not permitted at present in talking of organic compounds. If this should turn out to be true, then to say that the average of nitrogen changes requires energy would be like saying that after an assisting engine had been fastened to the rear of a freight train and was actually aiding its motion, the freight train required power to pull the engine along. It is subsequent changes of the carbon molecule rather than of the nitrogen molecule that should involve considerable changes in energy levels, owing to there being a greater range of valence change.

Fifth, an analogy to the energy change in the formation of higher nitrogen compounds may be found in the energy change when a simple non-nitrogenous carbon compound of high energy content condenses to form a carbohydrate, and without question the average free energy change of this sort is small, and of a negative character. We have the prominent instance of 6 molecules of HCHO polymerizing to form 1 of glucose. The heat of formation of HCHO according to



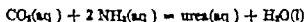
has been determined only quite recently by von Wartenberg and Lerner-Steinberg⁹ to be $-27,800$. The heat of formation of glucose is $-302,000$. From this we see that about 150,000 calories are liberated during this polymerization, an enormous amount which approximates one-half the heat of formation and one-quarter the heat of combustion of glucose. The conclusions are not different when free energies are considered in place of heats of reaction. Certainly the standard free energy of HCHO would be only a trifle different from the heat of formation of HCHO, but let us assume that these are equal. The value of the standard free energy of glucose has been determined from unpublished calculations by the author to be $-219,700$. The calculations were made by the entropy method with the use of the very excellent specific heat data containing thirty experimental points on a temperature scale which ranged from 19.1° to 287.2° absolute, obtained by Simon.¹⁰ Consequently in the polymerization reaction, ΔF° is about $-72,000$. In the plant the substances

⁹ von Wartenberg, H., and Lerner-Steinberg, *Z. Ang. Chem.*, 1925, xxxviii, 591.

¹⁰ Simon, F., *Ann. Phys.*, 1922, lxxviii, 241.

would hardly be in their standard states, but this value gives a good idea of the magnitude of ΔF when the reaction takes place in somewhat concentrated solutions. However, since there are six reactants to one resultant, the concentration of HCHO need not be very dilute before ΔF would become positive, in which case, true enough, energy would be needed for polymerization. In every instance of polymerization or condensation the effect of dilution is the same, that is, the greater the dilution the more positive does ΔF become. But the plant can very simply overcome this by removing the end products so promptly that ΔF would be negative, and presumably this occurs. Thermodynamics cannot testify that the organism does this, but the very fact that the greater number of reactions in plants go merely with the aid of non-energy-supplying enzymes or catalysts, strongly supports the view that the plant must carry on its reactions at concentrations where free energy would not be required.

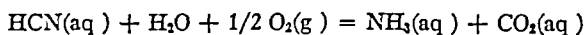
Sixth, the standard free energy of formation of a higher compound of nitrogen, carbon, hydrogen, and oxygen is known. For urea, ΔF° is $-47,280$. Thus a great deal of free energy is yielded in the formation of this higher compound. When urea is formed, not from its elements, but from two simple compounds, as in the equation



ΔF° is 5030, indicating that at standard conditions free energy is required. However, so long as urea is removed rapidly enough, then no matter how dilute the concentration of the reactants, the reaction will require no free energy. This particular reaction is typical of the reactions which ammonia may undergo with another compound to lose water, and shows that probably the standard free energy change in such condensations is small. Yet even assuming that this reaction when it occurs in a plant is at such a concentration as to require free energy, the free energy required in that event might well be drawn from a supply incidentally provided during the fixation of nitrogen into ammonia, and so the total free energy expended still remain negative.

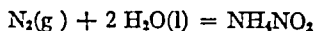
Returning to the thermodynamic argument, there are other reactions for the free energy yielding fixation of nitrogen. $\text{HCNO}(\text{aq})$ has a standard free energy of $-29,000$, urea, $-47,280$, and $\text{HNO}_2(\text{aq})$,

—13,070 These compounds may spontaneously form on condition that hydrogen gas is a reactant. Were the hydrogen, and therefore part of the oxygen, derived from water, as in the case of nitric acid discussed originally, then nitrous acid would be the only compound not requiring free energy for its formation, and this would be the case only at quite high dilution. In the cases of $C_2N_2(g)$, $CNI(s)$, $HCN(aq)$, $NO(g)$, $NOCl(g)$, $NO_2(g)$, $N_2O_4(g)$, and $N_2O(g)$, the standard free energies are positive. Accordingly, energy would be required if nitrogen is fixed as hydrocyanic acid, or as any of these substances. Since for assimilation, however, these fixed forms would be changed by reacting with water, to either an ammonia form or a nitrate form, the net result would be a yielding of free energy. For instance, in the reaction



ΔF° is $-73,510$. The standard free energy of formation of HCN is $27,520$, consequently the net result of fixation is $-45,990$.

It would be profitable to consider a fixation reaction which has been the source of some confusion. The reaction for the hydrolysis of nitrogen by water,



has a positive standard free energy of $85,690$. Lewis and Randall (1923) have calculated from this that a pressure (fugacity) of nitrogen of about 10^{51} atmospheres would be required for ammonium nitrate to be formed at an equilibrium concentration of 10^{-6} M. G. Oddo¹¹ had measured (1915) the ionization of water in air. He found it surprisingly large and concluded that a considerable amount of NH_4NO_2 should be formed by the hydrolysis of nitrogen. Calculations show that he confused mechanism with equilibrium. Even were the water completely ionized, and $2 H^+ + 2 OH^-$ substituted for $2 H_2O$ in the above equation, the pressure of nitrogen required would still be 10^{23} atmospheres. Linhart,¹² who was among the first to apply free energy data to biological problems, attempted to determine the efficiency of nitrogen fixation by *Azotobacter*. From analogy

¹¹ Oddo, G., *Gazz. chim. ital.*, 1915, xlv, I, 395

¹² Linhart, G. A., *J. Gen. Physiol.*, 1919-20, ii, 247

with somewhat related compounds, he made an elaborate calculation of the standard free energy of mannite, which was at that time unknown, even approximately. In seemingly arbitrary fashion he used the above highly endothermic and free energy demanding hydrolysis reaction (except that $\text{NH}_4\text{OH}(\text{aq})$ was the resultant). While the free energy data of nitric acid and ammonia may not have been available at that time, the heats of reactions as approximations might have been considered. Of all the possible fixation reactions he may have correctly chosen the one employed by *Azotobacter*. This hydrolysis reaction possesses a large positive free energy because oxygen gas is not among the reactants. Falk and McGee¹³ have obtained evidence that this hydrolysis reaction takes place to some extent in the presence of metallic iron, which they say they believe supplies the energy. Their evidence is not very extensive. The reaction goes appreciably in the electric arc, since only a few volts are needed.

There is experimental confirmation of the contentions in this paper. Workers on the nitrogen fixing bacteria have themselves hesitated to deny that the wide ratio (of at least 1 to 50) of the nitrogen fixed to carbohydrate available might be narrowed were it possible to provide the organisms with more favorable conditions of environment. While experiments in general have not suggested that this ratio may be narrowed, Truffaut and Bezssonov¹⁴ now present evidence that in the presence of nitrogen fixing bacteria corn develops normally and reaches maturity in mediums devoid of organic nitrogenous matter, and that the ratio of the carbonaceous material excreted by the roots to the nitrogen fixed approaches 1 to 1. Some of the carbonaceous material would certainly serve to supply the carbon requirements of the organisms, in this way reducing the amount available for the supposed energy need in nitrogen fixation. And so, it is not at all obvious that the carbonaceous excretion functions as a source of energy. Since the carbonaceous excretion would contain about 40 per cent carbon, the ratio of excreted carbon to nitrogen fixed is only narrower. It is to be noted that in these experiments, in which according to the authors the fixation was probably done by anaero-

¹³ Falk, K. G., and McGee, R. H. *Chem and Metall. Eng.*, 1923, *xxix*, 224.

¹⁴ Truffaut, G. and Bezssonov, N. *Sc. Sol.*, 1925 *iv*, 3-53.

bic organisms (*i e Clostridium*), no sugar or carbohydrate excretions could be detected, but only organic acids (*i e malic*) The organic acids are precisely the substances some of which yield hydrogen with the liberation of free energy, as would be required were the equations presented in this paper to be employed

There is strong evidence¹⁵ that *Azotobacter* fixes nitrogen as ammonia

Still more relevant is the recent extensive evidence of Christensen-Weniger¹⁶ He finds that the process of fixation by the nodule bacteria of several species of legumes is exothermic or nearly so The energy requirements of the nodule bacteria were not met with by increased assimilating powers in nitrogen-fertilized legumes Whether some of the nitrogen was supplied as fertilizer or some by fixation, the final dry weight was almost the same and from the small excess of growth in the fertilized plants he was able to fix the upper limits of the energy supply He found the eventual requirements for the nodule bacteria so small as to be unimportant He was quite aware of the exothermic heat of formation of ammonia

If the nitrogen were fixed by the bacteria as nitrate, which might then be reduced in others parts of the host plant by means of radiant energy, this might in a measure explain the seemingly less efficient assimilation of nitrogen by the nodule organisms in pure culture, since there they would need more energy to reduce the nitrate

SUMMARY

Fixation of nitrogen even with liberation of energy or free energy, will take place if either oxygen gas or hydrogen gas, or other substances, especially gases, whose standard free energies are close to zero, are involved to form either nitrates, ammonia, or cyanide, not to speak of still other compounds It has been pointed out that there are two and only two general conditions where nitrogen fixation can require energy These are, first, if nitrogen reacts with some compound like water with an already high negative free energy of formation and where negligible oxidation of nitrogen would occur, second,

¹⁵ Kostyschen, S, Ryskaltshuk, A, and Schwezowa, O, *Z physiol Chem*, 1926, cliv, 1

¹⁶ Christensen-Weniger, F, *Centr Bakt*, 2 Abt, 1923, lvm, 41, *Chem Abstr*, 1925, xi, 2509

if the plant does not take advantage of working at concentrations where the process would yield free energy

If nitrogen fixation is exothermic and free energy yielding, how is the carbohydrate requirement of nitrogen fixing organisms to be interpreted? Are the experimental determinations of the carbon to nitrogen ratio purely circumstantial? Is further hope given to those who may experimentally try to narrow this ratio to where the carbon used is only for the carbon requirements of general metabolism, exclusive of fixation? Do not hypotheses concerning the fixation of nitrogen in the evolutionary process, which are based on the conception that energy is required, lose some of their significance? Does it not suggest that perhaps fixation is far more universal than is supposed among living forms, particularly among the higher green plants, and thereby give encouragement to those who may wish to demonstrate this experimentally? Does it not indicate that perhaps the function of fixation is often to obtain energy for use in general metabolism? Is the general carbohydrate metabolism of the fixation forms to be regarded as being merely extremely inefficient? Or most suggestive of all, is the carbohydrate serving some unobserved function?

The author wishes to express appreciation of criticism offered by Professor G N Lewis, Professor G E Gibson, Professor C B Lipman, and Professor L G M Baas Becking

STUDIES ON PERMEABILITY OF MEMBRANES

I INTRODUCTION AND THE DIFFUSION OF IONS ACROSS THE DRIED COLLODION MEMBRANE.

By L. MICHAELIS AND W. A. PERLZWEIG

(From the Laboratory of Research Medicine Medical Clinic The Johns Hopkins University and the Marine Biological Laboratory Woods Hole)

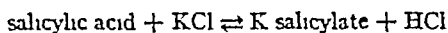
(Accepted for publication January 4 1927)

INTRODUCTION

When the different investigations on membrane potentials are taken into consideration, it seems that the theory of these potentials may be treated in three different ways. We may distinguish (a) phase boundary potentials, (b) mixed electrode potentials, (c) diffusion potentials.

The first theory assumes that the potential difference of each side of the membrane against the adjacent solution consists of a jump (a sudden, almost discontinuous fall or rise) of the potential at the boundary of two phases, the liquid being the one phase and the substance of the membrane acting as solvent for electrolytes being the other. The differences in the tendency of distribution of each single kind of ion between the two solvents which are in conflict with electroneutrality, produce the potential differences. This theory, first developed briefly by Nernst(1) and thereafter thoroughly elaborated theoretically and experimentally by Haber(2), has been applied to biological membranes by Beutner(3). The latter author also found different models in which he assumes the same mechanism, and at the same time, assumes that no essential part of the P.D. observed is produced within the membrane itself. He could reproduce membranes the potential of which depends on the concentration of any cation dissolved in the adjacent solution, and does not depend only on the concentration of one particular ion, as is the case with metallic electrodes and with the glass membrane, at least in certain kinds of glass such as Haber and

Klemensiewicz(4) used in their investigations Beutner's assumptions meet the following difficulties. In general, it can be shown that a membrane consisting of a water-immiscible phase and behaving as a solvent for the dissolved substances, cannot give any appreciable "concentration effect," that means any appreciable $P.D.$ when placed between two solutions of one electrolyte in different concentrations(5). In order to explain that such effects are brought about none the less, Beutner has to assume that in the oily phase there is a small amount of a weak acid (*e.g.* a trace of salicylic acid in salicylic-aldehyde), which is scarcely soluble in water, and that such a weak acid dissociates in the oil very strongly, like a strong acid or a salt, so that the reaction (written in terms of salts, not of ions)



will proceed amply in the direction \rightarrow , while it is known that this reaction in aqueous solutions practically goes on only in the direction \leftarrow . This assumption is necessary for Beutner's theory, but the experience of the last decade does not at all confirm such a behavior of weak acids in oils.

The second theory may be applied in such cases where the membrane has the character of an electrolyte-like substance (*e.g.* silicates, such as glass, permutit). One of the ions of the membrane substance may be an inert or "colloidal" ion such as the silicate ion, while the other ion which happens to be combined with the silicate (*e.g.* Na) shows what may be called an electrolytic tension towards the solution, like a metallic electrode. This ion, which forms a component of the solid silicate, may be partially exchanged for another ion present in the solution (*e.g.* H^+). Then the silicate behaves like a mixed Na and H electrode. The potential difference will depend on the kind and concentration of any such ions which are combined or are able to combine with the silicate in exchange for the ion originally combined with the silicate ion. Recently Horovitz (6-8) showed that this mechanism holds for the membrane potential produced with certain kinds of glass.

A third theory attributes the potential differences produced by membranes to the difference in the velocity of the single ions diffusing across the membrane. According to this theory, there is no abrupt

change in the potential at the boundary of the membrane but a gradual change of the potential from the one boundary towards the other, as is the case with a liquid junction potential when two different electrolyte solutions are in direct contact with each other, and by diffusion or convection a zone of gradual transition between the two solutions is interposed. So, this third theory is that of a diffusion potential. In a previous paper(9) the theory of such membranes as dried collodion or apple skin was developed on this basis.

It does not follow that these three theories are contradictory to each other. It may happen that two or even all three of these possibilities are combined in the same membrane. For instance, Beutner will probably not exclude the possibility of a diffusion potential within the membrane when it is in contact with two different solutions at the two sides. He only assumes that this diffusion potential plays quantitatively a negligible part in the total observed potential difference. On the other hand, Baur(10) endeavored to prove that the diffusion potential plays the predominating part and the abrupt change of the phase boundary potential may be neglected. Cremer(11), who was perhaps the first to emphasize the importance of these considerations, left it undecided which of these sources of potential differences plays the important rôle, emphasizing rather more the diffusion potential. The difficulty of the theory of diffusion potentials seemed to be that under any known condition the diffusion potentials, except the ones produced by acids or alkalis, are not great enough to give such a great effect as the membranes sometimes do. The differences in the mobilities of the different kinds of ions, except H^+ and OH^- ions, are not sufficient. We shall show, however, that in certain membranes these differences are enormously greater than the well known small differences in aqueous solutions. It was the aim of the author (9,12) to show that for a membrane such as collodion(12,d) the theory of diffusion potentials is sufficient to explain all known facts. The theory of mixed electrodes need not be considered because the chemical nature of collodion (and of some lipid membranes, such as the wax in apple skin) excludes this possibility. The substance of this membrane is not an electrolyte-like material nor does it consist of a cation and anion like a glass. However, the question may be asked whether the phase boundary theory may not be applied for collodion.

Now, for the usual permeable collodion membrane with its large pores, obviously the whole membrane effect is due only to these pores. There can be no doubt that in any of the well known dialysis experiments the dialyzing salts will go through the pores of the membrane and will not go through the substance of the collodion itself to any appreciable extent. Again, the transition from the usual large pored collodion membrane to the dried membrane is quite gradual. So it seemed most probable that the effect of the dried collodion membrane is due to the same mechanism as that of the usual collodion membrane. The dried membrane is to be regarded only as a practically obtainable limiting type of porous membrane with decreasing pore sizes.

Since the so called Donnan membrane potential has played a great part in researches on membranes in the last decade, it seems necessary to explain the relation of the Donnan potential to the different kinds of potential mentioned above. The difference is the following. All of the three mentioned theories attempt to trace the course of the potential from the one side of the membrane to the other. Two of the theories assume a more or less abrupt change of the potential at each boundary but no change elsewhere. The other theory assumes a gradual transition of the potential from the one boundary to the other. The total potential difference is made up by the sum of the single abrupt changes, or the integral of the single differential changes, according to the assumption. The Donnan theory is not concerned at all with the course of the potential across the membrane, it only takes into consideration the difference between the two sides of the membrane, and it can be applied to any case where one kind of ion, which is present only on the one side of the membrane, is permanently not present on the other side. Donnan stated as the necessary condition for the possibility of such a case occurring the non-diffusibility of one kind of ion across the membrane, i.e. the lack of any mobility across the membrane. However, the same condition may be the limiting case either for very low mobility of this particular ion in the membrane, or for a very low solubility of this ion in the membrane. In either case the liquid on the other side of the membrane remains permanently free from this particular kind of ion and the conditions of the Donnan equilibrium are fulfilled. In such a case, where either the mobility or the solubility of an ion within the membrane is really

zero, there is an abrupt change of the potential at the boundary of the membrane. However, the Donnan theory does not consider the course of the potential at all, and it deals only with the difference of the potential between the two sides. Neither does the Donnan theory concern itself with the mechanism of the impermeability of this particular ion. It holds at least for the limiting cases of two quite different mechanisms: insolubility and immobility. In reality, however, not only do the limiting cases occur, but also cases where either the solubility or the mobility of the ion in the membrane is only diminished and not completely abolished. Thus our system includes the Donnan potential as a limiting case among different possibilities.

It is also possible to develop the theory of the porous membrane upon the assumption of *different phases*. Certainly, the solubility, the activity, the osmotic pressure of any dissolved substance, and the vapor pressure of the solvent, are different in the bulk of the solution from those obtaining within the capillary pores and channels. One could speak, therefore, of a coefficient of distribution or partition, between the bulk and the capillary space, of any particular substance which is a common component of both. However, such an attempt would lead to great difficulties and is of no advantage whatever. The capillary spaces should not be considered as phases separated by a sharp boundary from the bulk phase, but one should rather conceive the capillary spaces as being analogous to the surface layer of any phase in contradistinction to the bulk. Thus the entire surface of all the capillaries of a sieve-like membrane is an enormously enlarged surface of the solution. We emphasize this idea in order to avoid a discussion as to whether the theory of phase boundary potentials ought to be applied to sieve-like membranes.

The problem of the potential differences produced by membranes between two solutions is closely connected with the problem of the permeability of the membrane. In the various attempts to apply the different theories to the selective permeability of the membranes of living cells, the conception of a lipid membrane as a solvent had been chiefly used, because of the influence of the well known studies of Overton. Here the membrane was assumed to be a homogeneous phase with sharply defined boundaries interposed between the inner phase (the protoplasm) and the solution in contact with the exterior

of the cell But since this theory was not satisfactory for every case, the idea of a mosaic membrane has been suggested by Nathansohn (13) This is supposed to be a porous membrane, a sieve consisting of a framework of a lipoid the pores of which are filled by an aqueous solution Here we have the sieve-like membrane which only differs from the collodion membrane insofar as the substance of the framework is not quite inert but participates in the permeability for certain substances which are soluble in it In any case, in order to separate these two alleged effects of the mosaic membrane we must first study the effect of a sieve membrane with an inactive framework in which the whole problem of permeability depends on the pores For that purpose the dried collodion membrane seems to be the almost ideal model, because no other porous membrane of sufficient mechanical resistance (clay, etc) can be obtained so easily with pores as small as in collodion M Traube's copper ferrocyanide membrane suffers from the lack of mechanical strength and requires always the presence of the membrane-forming substances, CuSO_4 , on the one side and Na ferrocyanide on the other This complication makes it almost impossible to study the properties of such membranes with those methods which proved the most suitable and simplest in the case of collodion membrane Collander (14) has recently made a very exhaustive study of the copper ferrocyanide membrane

1 The Problem of Direct Diffusion Experiments

In a series of experimental studies on the dried collodion membrane (12, *d, g, h, i*) it has been shown that this membrane is considerably less permeable for anions than for cations A first attempt at a theoretical treatment of the properties of such a membrane has been made in a previous paper in this journal (9)

The assumption of a relatively small permeability for anions was founded at the outset of these studies upon the following interpretation of the E M F of concentration chains When two solutions of one electrolyte in different concentrations are separated by a dried collodion membrane, a potential difference is established which in the best cases reaches the theoretical maximum value of an ordinary concentration chain with electrodes reversible for the cations The simplest inter-

pretation of this effect was the assumption that the anions are not, or at least are much less, mobile in the pores of the membrane than the cations. Such an assumption must, of course, be proved by direct diffusion experiments. These diffusion experiments are the subject of this paper.

In some of the previous papers (12, *d*, *e*) diffusion experiments have already been described which fairly well confirmed the assumption, but these experiments have not been perfectly satisfactory. The difficulties consisted in the following circumstances. In the first place, when the membrane was made thick enough to resist the mechanical strain in such an experiment, the time required to yield quantities of the diffused ions sufficient for chemical analysis was very long, weeks, even months. Very often the membrane did not retain its original properties for such a long time, and many experiments were spoiled in this way. On the other hand when the membrane was made thin enough to allow a sufficient diffusion in 1 or a few days, the lack of mechanical resistance spoiled many experiments. The membrane became leaky and the leaks could be shown sometimes simply in the ordinary macroscopic way, sometimes the leaks were relatively small so that they were not manifested macroscopically, but produced a potential difference between two KCl solutions, 0.1 and 0.01 N, much smaller than the expected maximum value. This value is, theoretically, as has been shown (9), 55 millivolts, and good membranes, give, in fact, a potential difference of 50 to 53 millivolts. Leaks not large enough to be visible macroscopically become manifest by a drop of this potential difference sometimes down to 25 millivolts or much less. Nevertheless, selecting the good experiments, it could be shown, that HCl diffuses against pure water across a membrane extremely slowly, while HCl and KCl exchange cations relatively quickly across the membrane. Other arrangements for diffusion experiments gave similar results. But all of the results so far obtained are only qualitative and not really satisfactory. In order to obtain reliable results a kind of membrane had to be employed which was permeable enough to give a measurable amount of diffusion in a few days, and which, on the other hand, retained its great difference of behavior towards cations and anions, and which also retained its properties during the time of the experiment. The authors finally

succeeded in obtaining the required kind of membrane by selection of a suitable kind of collodion and method of making the membrane

2 *The Method of Preparing and Standardizing the Membrane*

The general method of preparing this kind of membrane consists in pouring some solution of collodion into a cylindrical glass vessel, letting a film of collodion form adhering to the glass wall, permitting it to dry to a certain degree, and then, in distinction to the way in which the ordinary collodion membrane is made, not to detach it from the wall by means of wetting it with water, but simply by means of forceps or the fingers. The drying has to proceed rather far to allow this detachment. The best way is to wait until the detachment begins spontaneously. After the membrane has been pulled out of the glass vessel it must be dried further, for at least a day, in the open air. Several variations in this method are possible and we shall describe the one which seemed the most convenient.

When different kinds of collodion, even when dissolved in the same solvent medium, are employed, the properties of the membranes may vary to a great extent. The different samples of membranes made up from the same collodion solutions behave, though not completely, relatively fairly uniformly. As the best method of characterizing such a membrane the measurement of the potential difference between an 0.1 and an 0.01 *M* KCl solution separated by the membrane may be recommended. This may be called the *concentration potential* or the *Co P* of the membrane (12, 1). Thus *Co P* depends to a considerable extent on the kind of collodion used. At least ten different samples of collodion were used, such as "parlodion," "commercial gun cotton No. 1," and several samples of nitrocellulose of different nitrogen content. None of these gave really good results. Some of them gave at times membranes with a relatively high *Co P*, such as "gun cotton," but were so poorly permeable (which could also be shown by the very poor electric conductivity in an aqueous solution of some neutral salt) that they were useless for diffusion experiments. Others had a better permeability but had such a low *Co P* (between 25 and 40 millivolts) that the required specific properties of the membranes were not obtained. Such membranes showed, in

fact, only relatively small differences in permeability for anions and cations. For instance, an 0.1 N solution of HCl did not diffuse into pure H_2O across such membranes much slower than it did into a KCl solution. The one sort of collodion which was found satisfactory for these purposes was "Celloidin-Schering." Membranes may be made up either in a flat form, which will be more fully discussed in a subsequent paper, or in the form of bags. For diffusion experiments the bag form seemed to be preferable on account of the greater surface, though for many other purposes the flat membrane is preferable. The following method turned out to be most suitable for the required purposes.

5 gm. of commercial celloidin shreds, Schering previously dried in the air or by short washing with absolute alcohol, are dissolved in a mixture consisting of 75 cc. of absolute alcohol and 25 cc. of anhydrous ether. The process of solution may require several days and is accelerated by frequent gentle shaking.

Suitable tubes, such as 50 cc. round bottom centrifuge tubes, are filled with the above collodion solution, permitted to stand covered until the air bubbles rise to the top and disappear, after which the greater portion of the collodion is poured off leaving about 5 cc. in the tube. This residual amount is then carefully distributed in as uniform a layer as possible by slow rotation and warming in the palms of the hands. After several minutes of such rotation and evaporation of the solvents, the tube is placed in a clamp in an inverted position, mouth downward, and permitted to drain. As stated above, it is best to leave the tube undisturbed until the membrane begins to detach spontaneously from the glass. This may require under various atmospheric conditions 2 to 4 hours. The rim of the membrane is now cut with the point of a knife and it is removed by careful and gentle pulling. Special care must be exercised not to exert too great a strain upon the bottom of the membrane, so as not to stretch it too much at that place. At this point the membrane is still quite elastic, and the distortion due to the manipulation of detachment from the glass may be corrected by gently blowing into it several times during the next 5 to 10 minutes. The bags are now placed upon a clean surface and allowed to dry in free air for about 1 or 2 days.

It will be observed that at the end of this drying period all mem

branes show a greater or lesser degree of shrinking and wrinkling. The wrinkling can be partially prevented by repeated blowing during the process of the drying. The degree of this shrinking appears to depend upon the extent of drying previous to the removal of the bag from the glass tube, and upon the thickness of the collodion layer. As a rule longer drying within the glass tube and the thinner layers yield the smoother membranes. It may be noted, however, that a moderate degree of shrinking does not impair a membrane for the purposes described. A membrane which is entirely smooth is as a rule not very resistant to mechanical strain. On the other hand, strongly shrunk and wrinkled membranes may be unserviceable because of lack of uniformity of shape and texture. But the characteristic properties of permeability, conductivity, in a given electrolyte solution, and of the concentration potential seem to depend to only a small extent upon the differences in the amount of shrinking and on the relative shape. It is true that the degree of shrinking is not entirely without effect. Recently Liesegang (15) reported that collodion membranes, when prevented from shrinking during the process of drying, are much more permeable than the shrunk ones. This fact has been known to the authors for a long time, though it has not been expressly published, since the interest was directed towards obtaining the limiting case of membranes with the narrowest pores possible. It has also been known to the authors, *e g* that a collodion membrane made by impregnating a filter paper bag (extraction shells of Schleicher and Schull, or simply filter paper) is much more permeable than the membrane without a skeleton, even when completely dried. The *Co P* of such membranes never exceeded about 40 millivolts. For that reason this kind of membrane was abandoned by the authors, though it may be useful for other purposes.

A completely dried membrane is a perfect electric insulator and is highly electrified by gently rubbing it against the hair. Not being easily wetted by water, it retains its electric charge a very long time, even in a humid atmosphere. This property of an electric insulator may be emphasized because it shows that the subsequently described properties of permeability and electric conductivity are due to the pores and their contents and not to the chemical or physical nature of the solid nitrocellulose substance.

A quantitative expression of the most important characteristic property of these membranes is obtained by measuring the concentration potential (*Co P*) between an 0.1 *N* and an 0.01 *N* KCl solution separated by the membrane. A suitable arrangement for this measurement is shown in Fig. 1. The advantage of KCl solutions is chiefly in the lack of any diffusion potentials against the solutions of the calomel electrodes. As was stated above the best membranes will show a *Co P* at room temperature of 50 to 55 millivolts. This

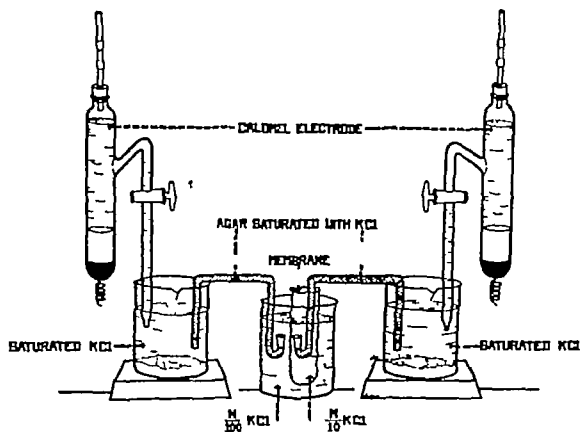


FIG. 1

Co P may be reproduced on the same day for any membrane within a fraction of a millivolt, and within a longer period of several days or even weeks, the *Co P* of the same membrane will not vary as a rule more than 3 or 4 millivolts. The best way of preserving the membranes is to immerse them in and fill them with distilled water.

Although the electric conductance of the membrane immersed in a solution of electrolytes does not come strictly within the scope of this paper, we cannot avoid touching upon this subject. The

resistance of such a system is different, of course, in the direct and alternating currents for well known reasons. The resistance which interests us from the standpoint of the permeability of the membrane for ions is the ohmic resistance to a direct current, since the magnitude of the conductance in a given electrolyte solution is in the sense of Nernst's theory a certain function of the diffusibility of the ions.

The conductance of a membrane may be approximately measured in the following simple manner using the arrangement shown in Fig. 1. The entire system is first made up *omitting the membrane*, filling the beaker with 0.01 N KCl. Now the potential difference of the system between the electrodes should be equal to zero. Being assured that such is the case, we establish by means of the potentiometer a certain potential between the electrodes of such magnitude as to produce currents of a given strength, *e.g.* 1.5×10^{-7} amperes. The current intensity is measured by means of the galvanometer, the sensitivity of which had been established by previous calibration. From the current intensity, I , and the applied EMF , read from the potentiometer, the resistance, R , may be easily calculated using the equation

$$I = EMF / R$$

For instance, with our Leeds and Northrup enclosed scale and lamp galvanometer, a deflection of ten lines on the scale indicated a current of 1.5×10^{-7} amperes. The resistance of the whole system without the membrane with a 0.01 N KCl solution in the beaker was about 3000 ohms. On inserting the membrane this resistance was increased by 600 to 8000 ohms or more, depending upon its relative smoothness and thickness.

It was surprising to find that such relatively high conductance was compatible with such highly pronounced specific influence on the relative mobilities of the cations and anions. To produce such differences in the mobilities, as are described later in this paper, we must assume extremely narrow pores. The high conductance of the membrane indicates that the number of these fine pores must be very great. Thus, for instance, for membranes prepared from a certain brand of gun cotton and which yielded about the same *Co P* effect, the resistance, under the same conditions, was found to be enormously greater, up to several million ohms, showing that in such membranes

the number of pores was correspondingly smaller, while the size of the pores upon which the $Co P$ depends is probably the same. On the other hand, we have observed membranes prepared from other kinds of collodion, *e g* parlodion, yielding much lower $Co P$ values (about 25 to 30 millivolts) with a higher resistance than in the celloidin membranes. Such membranes probably have larger but fewer pores. Hence the usefulness of our celloidin membranes is probably due to a large number of very fine pores. The size of these pores is apparently within the size order of larger single molecules, for, as shown in a previous paper (12), non-electric molecules in which hydration and electric charge do not play an appreciable rôle, diffuse through these membranes at a rate corresponding to their size. For instance, urea diffuses relatively readily, while the sugars do not diffuse at all, and with aliphatic alcohols the rate of diffusion decreases rapidly with increasing molecular size.

3 *Methods of Analysis*

The methods used were Potassium was determined by the method of Kramer and Tisdall (16) which was slightly modified for our purposes as follows. After suitable evaporation the whole or an aliquot portion of the solution containing about 0.3 to 0.05 mg. of K in a volume of 2 cc. was precipitated with an excess (2 cc.) of the cobalti-nitrite reagent, centrifugalized after standing for several hours, the precipitate collected on a small asbestos filter and washed on the filter several times with a dilute (approximately 0.01 *N*) magnesium sulfate solution. This solution was used to prevent the colloidal dispersion of the precipitate which occurs on washing with distilled water. The asbestos mat and precipitate were quantitatively removed to a small flask and titrated with *N*/50 permanganate and oxalate in the presence of an excess of sulfuric acid as given in the original method. Frequent checks showed that with this procedure we could determine with an accuracy of about 2 to 5 per cent analogous quantities of K in known solutions of KCl in the presence or absence of the other salts used in our experiments.

The chloride ion was determined by the well known Mohr method using the following procedure. Since the amounts to be determined were almost always extremely small the solutions were cautiously evaporated to dryness and the residue dissolved in 1 or 2 cc. of a 10 per cent K_2CrO_4 solution. (The chromate solution had been previously treated with a little $AgNO_3$ solution to remove traces of chloride present, and after standing for a day or 2 filtered.) The titration was carried out with *N*/100 $AgNO_3$ solution using microburette with a fine tip. The end-point of the titration the first appearance of a clear brown

tint, is best observed when the titration is performed in a white porcelain dish or in a glass vessel standing directly on a white surface. The excess of AgNO_3 necessary to produce this perceptible red brownish shade was found to amount, under these conditions, to about 0.02 to 0.05 cc (a small drop) of the 0.01 N solution, which amount must be subtracted from the titration figure.

4 *The Diffusion Experiments*

Several groups of experiments were carried out as follows

I 0.1 M KCl against H_2O Table I, A

0.1 M KNO_3 " 0.1 M NaCl, Table I, B

0.1 M KCl " 0.1 M NaNO_3 , " I, C

II The same experiments were carried out, but in this case each membrane was used for repeated diffusion experiments with increasing periods of time of diffusion. The solutions outside and inside the collodion bag were renewed at the end of each period, Table II, A and B

III In Table III are detailed experiments using the same salts as above but in much higher concentrations, 0.5–1.0 M

IV Three experiments were carried out in which the membranes separated 0.1 M electrolyte solutions with the cation (K^+) in common but with different anions (Cl^- and NO_3^-), as shown in Table IV

V Table V shows a series of experiments in which the molar concentrations of the two electrolytes separated by the membrane were deliberately chosen in the ratio 1:5 with the chloride having the higher concentration. In this arrangement the p.d. of the Na^+ solution against a K^+ solution is almost completely abolished, as will be more fully explained in the discussion of the results

VI The experiments detailed in Table VI are presented to show the effect of low Co P values of the membranes and also to show that some kinds of collodion are not suitable for the purposes of approaching the characteristics of an ideal semipermeable membrane for ions. The membranes used in Series I to V were prepared for Celloidin-Schering, whereas the membranes used in this series were made from other commercial brands of nitrocellulose

TABLE I

A. Diffusion of 0.1 M KCl against H ₂ O							
Membrane No.	C/P of membrane		Time of diffusion	Diffused amounts		Ratio K:Cl	Diffused K per day (24 hrs.)
	Before	After		K	Cl		
	mM		hrs	moles $\times 10^{-4}$			moles $\times 10^{-4}$
1	53.8		24	2.0	4.8	0.4	2
		42.0	42	1.6	3.5	0.5	0.92
2	51.5		70	5.6	5.1	1.1	1.3
3	49.8	46.7	70	3.6	3.2	1.1	1.2
B. Diffusion of 0.1 M KNO ₃ against 0.1 M NaCl							
1	53.0		24	9.1	1.8	5	9.1
2		37.5	42	23.8	3.2	7	13.6
3	52.0		24	20.3	2.0	10	20.3
4		49.2	42	36.4	3.0	12	20.8
5	52.0	54.3	44	91.6	1.8	51	20.7
6	47.5	54.3	44	59.6	1.5	40	11.5
7		50.2	92	11.0	1.0	11	2.9
8		20.2	120	35.0	1.0	35	7.0
9		48.5	120	16.4	1.9	9	3.3
C. Diffusion of 0.1 M KCl against 0.1 M NaNO ₃							
1	53.0		24	3.6	0.8	5	3.6
2		53.5	42	6.1	0.6	10	3.5
3	53.5	51.2	70	13.8	1.4	10	4.7
4	53.5	50.0	70	65.5	4.9	13	22.5

In Series A where KCl diffused against pure water the diffused amounts of K⁺ and Cl⁻ are extremely small and, within the experimental error equivalent to each other.

In Series B and C where the membrane separated two electrolyte solutions, the amount of K⁺ diffused is much higher and the ratio of diffused K⁺:Cl⁻ is within the range of 4 to 20.

TABLE II
Progressive Diffusion Experiments with 0.1 N Solutions

A KCl against NaNO ₃						
Membrane No	C ₀ P of membrane		Time of diffusion	Diffused amounts		Ratio K Cl
	Before	After		K	Cl	
1	51 5	52 2	3	1 6	<1	—
			6	3 3	<1	—
			12	7 2	1 7	6 6
2	51 0	37 0	3	4 6	<1	—
			6	10 8	1 7	6
			12	24 2	7 4	3
3	52 4	48 8	2	14 2	1 8	7 5
			4	31 0	3 2	9 7
			8	70 0	8 0	8 8
			12	94	13 2	7 0
4	53 7	52 7	2	6 6	1 2	5 5
			4	15 0	1 2	12 5
			8	33 0	2 6	12 7
			12	50 0	5 1	10 0
5	52 7	47 4	2	11 6	2 4	5 0
			4	23 4	5 2	4 5
			8	59 0	13 9	4 2
			12	96 0	16 0	6 0

B KNO ₃ against NaCl						
1	51 0	41 0	3	14 3	4 3	3 3
			6	32 2	3 2	10
			12	67 0	9 2	7
2	53 1	46 5	2	11 6	1 2	10
			4	25 0	1 5	16 6
			8	56 0	2 6	21 5
			12	85 0	5 2	16 3
3	50 2	41 0	2	28 3	5 6	5
			4	63 5	9 4	6 6
			8	150	30	5
			12	214	52	4 1

In this series each membrane was used for a number of successive experiments, with progressively increasing diffusion time. Again, the value of the ratio of diffused K/Cl approaches about 10, sometimes remaining almost the same in the successive experiments, sometimes with a tendency to an increase followed by a decrease in the ratio.

TABLE III.

A. Diffusion of 0.5 M KCl against H ₂ O						
Membrane No.	Co P of membrane		Time of diffusion	Diffused amounts		Ratio K:Cl
	Before	After		K	Cl	
	mv		hrs	mole $\times 10^{-4}$		
1	54.6	50.8	20	1.0	0.8	1.2
2	55.0		92	21.5	18	1.2
B. Diffusion of 1.0 M KCl against H ₂ O						
3	50.8	50.7	48	0.95	0.85	1.1
4		46.3	216	1.13	1.62*	0.1 appr
C. Diffusion of 0.5 M KCl against 0.5 M NaNO ₃						
1		42.5	20	8.5	7.0	1.2
2		42.6	92	13.8	7.0	2.0
3		37.2	360	160.0	155.0	1.0
4		45.2	120	31.0	23.5	1.3
5		51.7	120	37.0	40.4	0.9
6	51.8	51.2	120	84.0	62.5	1.3
7	54.6	50.8	120	96.0	58.0	1.7

In the above experiments with 0.5 or 1.0 M solutions instead of 0.1 M the ratio of diffused K⁺:Cl⁻ is decidedly lower being approximately the same whether the diffusion takes place against water or another electrolyte.

* Analysis uncertain

TABLE IV
Diffusion of Anions Only

Diffusion of 0.1 M KNO ₃ against 0.1 M KCl				
Membrane No.	Co P of membrane		Time of diffusion	Cl ⁻
	before	after		
	mV		days	mM
1	51.5	49.7	12	0.0048
2	51.2	51.0	12	0.0068
3	51.2	39.6	12	0.400

Experiments 1 and 2 show the extremely slight amount of Cl⁻ ion diffused in a period as long as 12 days. Experiment 3 shows that a much larger amount of Cl⁻ ion diffuses when the character of the membrane has been impaired as shown by the relatively large drop in the Co P to 39.6 millivolts.

TABLE V

Diffusion of 0.05 M KNO₃ against 0.25 M NaCl (No P.D. between the Separated Solutions)

Membrane No	Co P of membrane		Time of diffusion	Diffused amounts		Ratios	
				K	Cl	K/Cl	U_{K^+}/V_{Cl^-}
	<i>before</i>	<i>after</i>	<i>hrs</i>	<i>mols × 10⁻⁴</i>			
1	52.4	54.3	196	81	60	1.4	7
2	50.3	52.4	196	47	8.0	5.9	30
3		50.6	240	52.2	3.2	16	80
4	49.3	47.8	196	25	9.6	2.6	13
5	52.4	54.3	196	81	60	1.4	7
6	48.6	47.2	196	34.2	22.0	1.6	8
7		46.0	144	14.8	0.5	30	150
8	57.0	41.5	144	5.0	1.5	3.3	16
9	56.5	39.5	144	7.6	1.1	7	35
10	50.3	37.2	196	48	124.0	0.4	2
11	52.0	24.8	144	7.3	2.0	3.7	18
12	51.0	23.5	144	15.7	14.9	1.00	5

Though the concentration fall of chlorine is five times as great as that of potassium, the amount of diffused K^+ is always greater than that of Cl^- , except in experiment No. 10. The last column shows the mobility ratio of K^+ and Cl^- , referred to the same concentration gradient. This ratio is always greater than 1. The exceptionally high value in No. 7 may be due to an error in the particularly low amount of Cl^- . The experiments are arranged according to the Co P values after the experiments. However, the figures of the last column do not follow the same order. It should be noticed that the Co P of many membranes became much smaller after the experiment, showing that the character of the membranes are somewhat changed during the diffusion. This may explain the differences in the ratios.

TABLE VI

Diffusion Experiments with 'Gum Cotton' Membranes of Lower Co P 0.1 M KNO₃ against 0.1 M NaCl

Membrane No	Co P of membrane		Time	Diffused		K : Cl ⁻
	Before	After		K	Cl ⁻	
	mm		days	mols × 10 ⁻⁴		
1	18.5	26.5	8	7.3	7.1	1.0
2	30.7	22.5	8	87.0	46.9	1.9
3	23.3	23.0	8	9.3	8.3	1.1
4	26.5	26.1	8	35.0	19.4	1.8
5	29.0	22.5	8	10.0	6.2	1.6
6	28.7	23.1	8	64.0	32.8	2.0

The same with "parlodion" membranes

7	20.0	21.5	8	1.94	2.3	1
8		16.6	26	2.6	2.6	1
9		19.1	26	13.5	8.4	1.6
10		24.3	26	15.8	9.0	1.7
11		23.8	26	23.3	14.0	1.7

This table shows, that in membranes characterized by a low Co P (probably due to a larger pore size) the K : Cl ratio approaches 1 and that the specific effect of the membrane on the mobilities of cations and anions disappears

Discussion of the Diffusion Experiments

It can be seen from the above tables that the diffusion of an electrolyte (KCl in Table I, A) is slower across the membrane into pure H_2O than into another electrolyte solution. Though the membranes are not uniform enough as to thickness and other properties to permit an exact quantitative comparison of experiments with different membranes, still it can be seen from Table I, that on the average the amount of K^+ diffusing per day is much less in A than in B or C. This comparison indeed is not very reliable on account of differences in thickness and other properties of individual membranes. However, what is certain is the fact that the amount of diffusing Cl^- corresponds to the amount of K^+ in the diffusion against water (A), whereas it constitutes but a small fraction of the K^+ in the diffusion against another electrolyte (C). In Table I, A, the amounts are so extremely small, that an exact agreement of the analyses cannot be expected. The error of the methods in these very small ranges tends always to yield high values for Cl^- . In any case the order of magnitude for the K/Cl ratio is 1:1, whereas this ratio in B and C is on the average 10:1, varying from 7:1 to 50:1. Only in two experiments in which particularly small amounts had to be analysed the ratio came down to 5:1. Here the error of the method may depress the value, and even if it does not, the ratio 1:1 is far from being reached.

Special attention should be drawn to the experiments shown in Table II, A. Here every membrane was used in a series of successive diffusion experiments with the same combination of electrolytes (KCl against NaNO_3) lasting for progressively increasing periods. First it can be seen that Membrane 2, for example, in which the *CoP* dropped in the course of the experiments from 51 to 37.0 millivolts, gives relatively low ratios for the diffusion of K and Cl, dropping down to 3, while the membranes with a higher and more stable *CoP* give ratios of about 7 to 12. In general, within the limits of error, this ratio is approximately the same, though not quite exactly so. It seems that these irregularities are greater than the limits of error in the analyses, and that the regularity and reproducibility of these experiments is not perfect.

The same remarks hold for similar serial experiments with 0.1 M

KNO_3 against NaCl in Table II, B. In general, the amount of potassium diffusing seems to follow a straight line plotted against the time, whereas the chlorine diffusing shows decidedly less regularity. This may be partially due to the greater difficulty in the quantitative determination of these minute amounts of chlorine, but we believe this is not the sole factor.

All of the above experiments were carried out with 0.1 M solutions. In higher concentrations this effect becomes less and less pronounced. So in Table III, with 0.5 M solution of KCl , the amounts of K^+ and Cl^- diffusing are approximately equivalent to each other, both when the diffusion takes place against H_2O and against a 0.5 M solution of NaNO_3 . It may be recalled that in such high concentrations the P.D. between two KCl solutions of different concentration vanishes (12, δ), to which problem we will refer in a later communication.

The experiments shown in Table IV in which the cation on both sides of the membrane is the same, but the anions are different, demonstrate that the anions diffuse with extreme slowness, even though the possibility of exchange is present. Membrane 3 of this series, the only one through which a much larger amount of Cl^- diffused, shows at the same time a considerable drop in its CoP during the experiments, while Membranes 1 and 2 maintained their original properties as evinced by only small change in their CoP values.

A special discussion is required for the experiments shown in Table V. Here an 0.05 M KNO_3 solution was diffusing against five times as concentrated a solution of NaCl . Thus the gradient of the Cl^- concentration across the membrane was five times as great as that of the K^+ ions. None the less, the amount of diffused K^+ is higher than that of Cl^- . If we assume that the velocity is proportional to the driving force, the specific velocities of K^+ and Cl^- can be calculated, in relative terms, by dividing the diffused amount of Cl^- by five. Thus we obtain the ratio of mobility of Cl^- and K^+ shown in the last column of Table V. The reason for carrying out this kind of experiment was the following. In the other experiments with equal concentrations of KNO_3 and NaCl , a potential difference is established. Any solution of a K salt shows a P.D. against any solution of a Na salt in equal concentration across the membrane, which in the best membranes reaches about 50 millivolts (12, δ). Now, the driving force

in the movement of a single species of ion through the membrane is the algebraic sum of the driving force resulting from the concentration difference of this ion on the two sides of the membrane and of the force of the electric field. It is not only the concentration fall which determines the rate of diffusion. Since the side with the Na solution is positive, there is, beside the osmotic force, an additional force, which is directed for positive ions from the Na solution towards the K solution and for negative ions in the opposite direction. Therefore, when the concentration fall for K ions from left to right is the same as the concentration fall for Cl ions from right to left, the total driving force is different for K and for Cl ions. The K ions are retarded, the Cl ions are accelerated by the electric forces. The specific mobility of an ion is its observed speed of movement divided by the driving force. But since the driving force is complicated, the calculation is difficult. The ratio of observed diffusion for K^+ and Cl^- does not represent the ratio of the specific mobilities of these ions. Now, by a suitable arrangement in the concentrations of the K and Na salts, the conditions can be made such that practically no $P D$ arises between the two solutions. This is the case when the concentration of the Na salt is about five to ten times as great as that of the K salt. The exact ratio necessary to bring about the complete abolition of the $P D$ depends on the individual $P D$ of a K-Na chain with the particular membrane. But for a ratio of concentrations 1:5 the $P D$ is in any case so low, that the electric force is negligible in comparison with the osmotic force. In fact, the $P D$ in the arrangement of the experiments of this series, Table V, was measured in several cases and found never in excess of a few (0.5 to 2.0) millivolts, whereas the same membranes, when interposed between a NaCl and a KNO_3 solution in equal concentrations, showed a $P D$ of 40 to 50 millivolts according to their individual properties and in agreement with the earlier findings with KCl-NaCl chains (12, d). Therefore, the driving force causing the movement of the Cl^- ions may be set simply five times as great as the driving force for K^+ . It is probable that the figure five is not quite correct, because neither osmotic pressure nor activity is exactly proportional to the concentration. However, these deviations are certainly within the ranges of the other errors in these experiments.

In this way, the "specific mobility" of K^+ and Cl^- , or at least the ratio of the two, is calculated in the last column of Table V. It may be emphasized that these ratios hold only for the conditions of this experiment, since the specific mobility of an ion within the membrane depends greatly on the conditions, especially on the concentration, to a much greater extent than it does in a free aqueous solution.

SUMMARY

The theoretical aspects of the problem of sieve like membranes are developed.

The method of preparing the dried collodion membrane is described, and the method of defining the property of a particular membrane is given. It consists of the measurement of the *Co P*, that is the p.d. between an 0.1 and an 0.01 M KCl solution separated by the membrane. *Co P* is in the best dried membranes 50 to 53 millivolts, the theoretically possible maximum value being 55 millivolts. Diffusion experiments have been carried out with several arrangements, one of which is, for example, the diffusion of 0.1 M KNO_3 against 0.1 M NaCl across the membrane. The amount of K^+ diffusing after a certain period was in membranes with a sufficiently high *Co P* (about 50 millivolts or more) on the average ten times as much as the amount of diffused Cl^- . In membranes with a lower *Co P* the ratio was much smaller, down almost to the proportion of 1:1 which holds for the mobility of these two ions in a free aqueous solution. When higher concentrations were used, e.g. 0.5 M solution, the difference of the rate of diffusion for K^+ and Cl^- was much smaller even in the best membranes, corresponding to the fact that the p.d. of two KCl solutions whose concentrations are 10:1 is much smaller in higher ranges of concentration than in lower ones.

These observations are confirmed by experiments arranged in other ways.

It has been shown that, in general, the diffusion of an anion is much slower than the one of a cation across the dried collodion membrane. The ratio of the two diffusion coefficients would be expected to be calculable in connection with the potential difference of such a membrane when interposed between these solutions. The next problem is to show in how far this can be confirmed quantitatively.

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PHOSPHATE ION AS A PROMOTER CATALYST OF RESPIRATION

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I

In other papers (Lyon, 1923-24, 1927) results have been reported which appeared to show that neutral solutions of sodium or potassium phosphate serve to catalyze the enzymatic production of CO_2 by plant tissues. The oxidising enzymes involved were those of *Elodea canadensis*, wheat seedlings, and potato tubers, the latter studied only in aqueous extracts. We shall now present additional proof of this promoter action through more careful analyses to determine the active component of mixtures of mono- and disodium phosphates.

The molar concentrations of the solutions which gave optimum results were somewhat high for the usual types of catalysis. The concentration most used was approximately 0.1 M, by which is meant a solution obtained by mixing 0.1 M monosodium phosphate with 0.1 M disodium phosphate. The complex nature of the components of such a solution suggested that some single element among them was the active, or at least the controlling factor of the catalysis. Since the ionization of even this concentration of the sodium phosphates is presumably complete, we are concerned primarily with the nature of the ionization products of phosphoric acid. An excellent statement of the conditions of equilibrium between $\text{H}_2\text{PO}_4'$, HPO_4'' , and PO_4''' is given by Holt, La Mer, and Chown (1925¹), from which it is apparent that for H_2PO_4 , K_1 is very large, K_2 is smaller, and K_3 , which determines the relative amounts of PO_4''' , is very small. These authors have also calculated the concentrations of PO_4''' in relation to pH over a wide range and have introduced the expression $p[\text{PO}_4''']$ which may

¹ Holt, La Mer, and Chown (1925) pp. 518 to 522.

be used to express the concentration of this ion just as $p[H^+]$ is used for the hydrogen ion

The relative concentration of PO_4''' is so low that it must be admitted that it is no higher than that of well known catalysts such as the H^+ ion. At pH 7.0 only 1/500,000th of the total P present is present in this ion. On the alkaline side of neutrality the relative concentration of PO_4''' increases rapidly and on the acid side it falls off. A plot of tables of Holt, La Mer, and Chown (1925²) shows that the graph of $p[PO_4''']$ (we prefer to use the form pPO_4) against pH is not a straight line but a curve between pH 6 and 9, which correspond to pPO_4 7.44 and 3.4, respectively. Thus pPO_4 will vary with both pH and the molar concentration of the acid or its sodium salts.

Similar statements could be worked out for the other ions of the solutions but it is this ion which proves to be related to catalysis through mathematically simple and exact rules.

As we shall show later, the concentration curve for the rate of CO_2 production by *Elodea canadensis* (Lyon³) in different concentrations of neutral phosphate solutions after an exposure of 1 hour does not afford the best data by which to test the conception of catalysis by the PO_4''' ion. At the two extreme concentrations other factors intervene to mask the real effect. When the concentration is low the element of rate of penetration of the phosphate into the living cells limits the observed effect at the end of 1 hour. At the higher concentration there is opportunity for a deleterious effect through either the osmotic effects or some other result of the presence of such a high concentration of salts. The intermediate data are too few to use.

Accordingly, we have performed the experiments necessary to provide data for the effect of change of pPO_4 through change of pH on the alkaline side of neutrality. Similar experiments were attempted for the acid side, but the presence of carbonates or bicarbonates gave rise to such an increase in the liberation of CO_2 that such readings are not comparable with those at a higher pH. In Fig. 1 are shown individual time curves of experiments on the alkaline side of neutrality. The significant values for our purpose are the levels at which each curve flattens out. These experiments were performed according to

² Holt, La Mer, and Chown (1925), p. 521

³ Lyon (1923-24), p. 302

the technique described in previous papers, the main apparatus being a suitable form of the Osterhout respirometer (Osterhout, 1918-19, 1919-20) Hence, the preliminary rise in most of these curves is

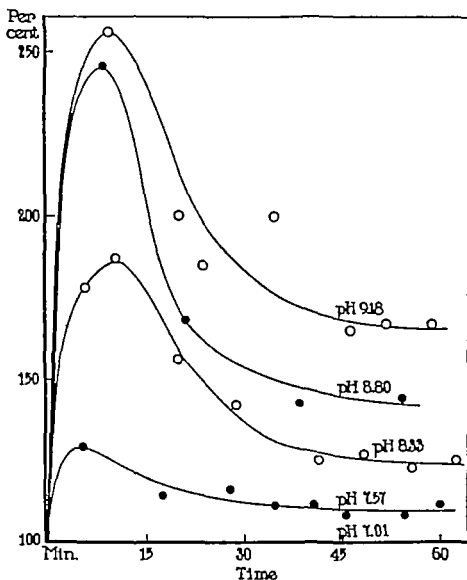


FIG. 1 The effect of alkaline phosphate solutions on the production of CO₂ by *Elodea canadensis*. Each curve represents a typical experiment in which the normal (taken as 100 per cent) is obtained with an 0.106 M neutral phosphate solution and followed by the application of phosphate mixtures of the same molar concentration but with the pH as indicated for each curve.

probably not due to a serious error such as the introduction of atmospheric CO₂ at the time of application of the alkaline solution. It is more likely that we have to do with a temporary change in equilibrium

within the system though there may possibly be a real temporary increase in rate of CO_2 production above that at which a level is attained

In Fig 2 are shown the mean values (of all experiments) of the rate of CO_2 production at the end of 1 hour, plotted against pPO_4 , the latter calculated from the tables of Holt, La Mer, and Chown, and against pOH . The solid line shows the regularity of the relationship between the PO_4''' ion and the rate of production of CO_2 . From the

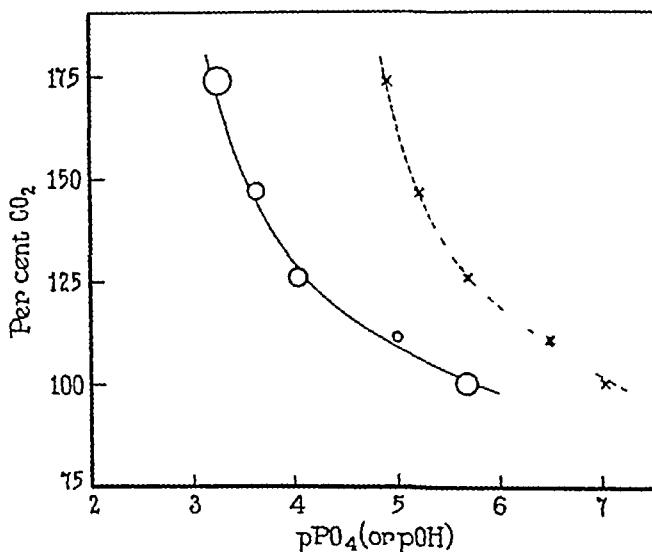


FIG 2 The mean rates of production of CO_2 by *Elodea canadensis* in 0.106 M alkaline phosphate solutions of different pH in relation to pPO_4 (solid line) and to pOH (broken line), after 1 hour

nature of the relationship between pPO_4 and pH it is to be expected that the relationship to pOH (broken line) should also be regular. We are attempting to show that the principal effect is that of the PO_4''' ion rather than the OH^- or H^+ ion.

The curve for the relationship to the PO_4''' ion resembles that of a hyperbola with the general equation (for these coordinates)

$$(\text{CO} - a)(\text{pPO}_4 - b) = K,$$

where a and b represent the fact that the asymptotes of this hyperbola may not be $\text{CO}_2 = 0$ and $\text{pPO}_4 = 0$ but $\text{CO}_2 = a$ and $\text{pPO}_4 = b$. The mathematical solution of this equation for the five measured points on

the curve was accomplished by a reliable method that depends upon the general method of least squares. The equation which results is

$$(\text{CO}_2 - 68.475) (\text{pPO}_4 - 2.13) = 114.43$$

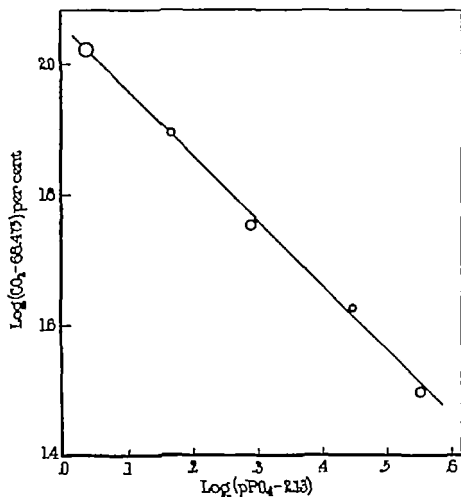


FIG. 3. The rates of production of CO_2 by *Elodea canadensis* in 0.106 M alkaline phosphate solutions of different pH in relation to pPO_4 . The ordinates are logarithms of per cent $\text{CO}_2 - 68.475$ while the abscissae are logarithms of $\text{pPO}_4 - 2.13$. The straight line is the plot of values calculated from the equation in the text.

The closeness of fit to the data may be seen in Fig. 3 in which the logarithm of the per cent CO_2 is plotted against the logarithm of pPO_4 . If the hyperbolic relationship holds the points should lie in a straight line in this type of plot. The straight line we have drawn represents the calculated equation while the points indicate the locations of the measurements.

The meanings of the constants a and b are interpreted as follows. Under the conditions by which the relationship was derived (μ with

the pH also increased) the one asymptote locates the point beyond which further increase in concentration of PO_4''' ions fails to give an increase in rate of CO_2 production, the other asymptote denotes the fact that the enzyme may function apart from PO_4''' ions to the extent of 68.475 per cent, where 100 per cent is the rate measured in neutral phosphate solution (0.1 M). Not much emphasis can be attached to these constants, however, since they were not obtained by changing only one variable—an impossible step in this work.

We may now observe the relation of this equation to the points on the concentration curve for *Elodea* (Lyon, 1923-24, Fig. 2). The horizontal form of the curve at molecular concentrations approaching the threshold of plasmolysis could not be expected to check with an equation derived from measurements at optimum salt concentrations. Some limiting factor may also prevent a close adherence to a hyperbolic relationship.

Likewise, the lowest concentrations might not exhibit the same relation. Here it is found that if the increase in molar concentration be thought of as a slow increase in concentration of PO_4''' ions (as is the case), the slope of the curve for CO_2 production is greater than that of Fig. 2 and is sensibly uniform over the first third of the graph. This linear, or very nearly linear relationship, which denotes direct proportionality, is typical of catalytic processes over the range of low concentrations and does not conflict with the relationship expressed by our derived equation. At the midrange of concentration, however, we should expect some conformity and such was found to be the case. The total difference between the K 's computed for the two known mid-points is only 1.5 per cent of the mean K .

This is as far as we can go in the analysis of our own data which seem to point to the PO_4''' ion as the effective catalyst of oxidising enzymes. It would be very desirable to obtain similar concentration curves for other plant material, by experiments involving change of concentration of the phosphate ion by changing first the pH and then the molar concentration. We plan to conduct such studies at a later date.

The results of Bode (1926), who sought to measure the dependence of respiration upon hydrogen ion concentration, show a qualitative agreement with our results in so far as his data may be converted into data for pPO_4 . His use of calcium and magnesium phosphates to regulate the pH introduces new variables affecting the exact concen-

tration of the phosphate ion and involving the influence of the metal. In general, however, the presence of higher concentrations of phosphate ion correlates with a higher rate of respiration as measured by the absorption of oxygen

II.

A further confirmation of the promoter action of the phosphate ion is afforded by the results of analyses of some existing records in the literature of other enzyme actions. Appropriate treatment of such records demonstrates the same hyperbolic relationship

The widespread practice of using phosphate buffers for controlling the pH of enzyme reactions has led to the statement of some records in a form which allows the transposition of either molecular concentration or change of pH into data for pPO_4 . Unfortunately it is common practice to report but a few "typical" series of measurements. Mean values of two measurements were found for some cases and of course these as well as data covering both change of pH and change of molar concentration at constant pH in the same type of experiments carry more weight.⁴

The method of procedure in the conversion of data into terms of pPO_4 will be described for only the first set of readings to be dealt with—those given by Smurnoff (1925) in a study of the effect of neutral salts on peroxidase. The enzyme was obtained from ground wheat seeds. The substrate was pyrogallol. The criterion of enzyme action was the amount of purpurogallin formed, estimated by titration with $KMnO_4$. The data for the effect of the phosphate solution of concentration $N/80$ were given for only one set of experiments, in the following tabular form

pH	3.5	4.0	4.98	6.5	7.1	7.5	7.86	8.3
Activity of enzyme, per cent.	1.9	2.84	5.79	100.0	135.0	148.16	150.53	175.07
pH.	8.7		9.3	9.5				
Activity per cent	183.64		173.86	139.68				

⁴ The measurements, however, are all recorded in terms of amounts of product after equal periods of time. That this is not always the proper measure of enzyme action was shown in the recalculation by Northrop (1924-25) of the results of Morgulis (1921) in a study of the kinetics of catalase action on peroxide.

From these data it is obvious that below pH 4.98 there is a powerful inhibition of enzyme action and above pH 8.7 there is a depressive action. These effects are undoubtedly due to a specific effect of the pH value and we can only use the intermediate data. The necessary data for a logarithmic plot in terms of $p\text{PO}_4$ is given in the following table. The $p\text{PO}_4$ was obtained by interpolation from a plot of the table of $p\text{PO}_4$ for various pH values.

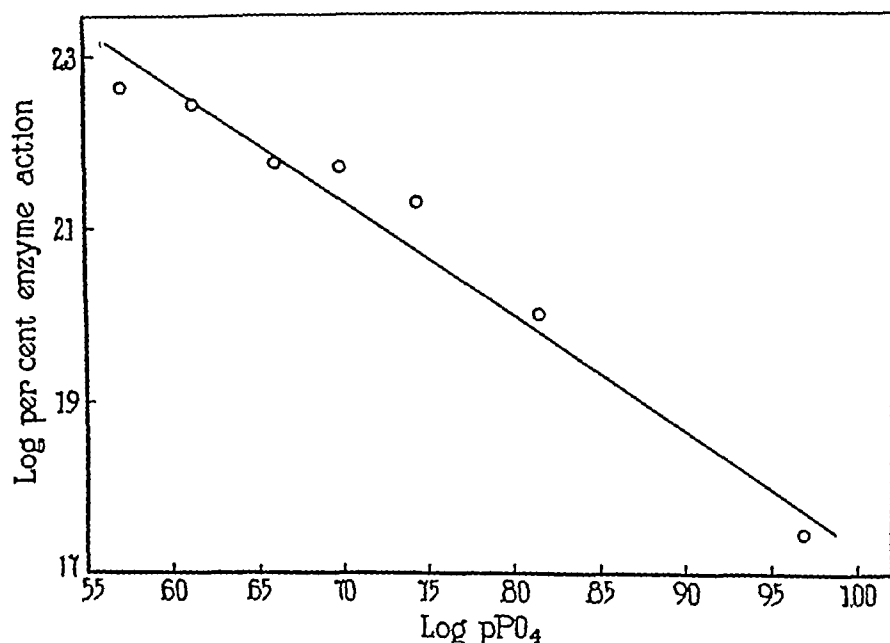


FIG. 4 The logarithmic plot of the relation of the concentration of $\text{PO}_4^{'''}$ ions to the percentage rate of oxidation of pyrogallol to purpurogallin by peroxidase. The straight line is the plot of the equation

$$(\text{Activity of enzyme}) (p\text{PO}_4)^{1.34} = K$$

The indicated points represent a single set of readings as reported by Smirnoff (1925)

pH	$p\text{PO}_4$	Log $p\text{PO}_4$	Enzyme activity in per cent	Log enzyme activity
4.98	9.43	0.9745	55.79	1.7465
6.5	6.51	0.8136	100.0	2.0000
7.1	5.53	0.7427	135.0	2.1303
7.5	5.00	0.6990	148.16	2.1707
7.86	4.58	0.6609	150.53	2.1775
8.3	4.10	0.6128	175.07	2.2430
8.7	3.72	0.5705	183.64	2.2640

Fig 4 shows the plot of these values. The points lie as near to a straight line as could be expected for a single set of readings. The slope of this line is not -1 , however but -1.34 . Therefore the equation is

$$(\text{Activity of enzyme}) (\text{pPO}_4)^{1.34} = K$$

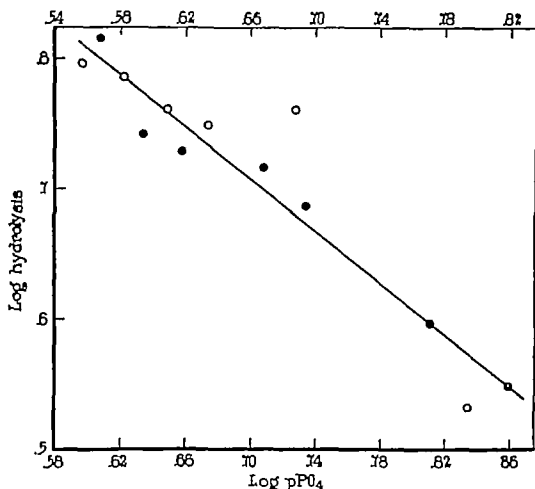


FIG 5 Logarithmic plot of the relation of concentration of $\text{PO}_4^{''}$ ions to the rate of hydrolysis of ethyl butyrate by pancreatic lipase in glycerin extracts of the pig pancreas. Data are taken from the work of Platt and Dawson (1925) who varied pPO_4 by change of pH. The straight line is fitted to the points (●) obtained from the readings for an experiment involving the use of low concentration of phosphate salts. The abscissae are those from the scale at the bottom of the figure and the equation for the line is

$$(\text{Hydrolysis}) (\text{pPO}_4)^{1.34} = K$$

The other points (○) are referred to the scale of abscissae at the top of the figure this scale differing from the lower one only by a displacement to the right by four units. These points were taken from a second experiment which differed only in the use of five times as much phosphate salts in the buffer solutions.

Smirnoff did not study in sufficient detail the effect of the concentration of phosphate solutions to warrant a similar test of the phosphate ion relations at constant pH. The method of calculation of $p\text{PO}_4$ under those conditions is fundamentally the same and will appear in connection with lipase analyses.

For another of the oxidising enzymes—laccase from alfalfa—we have found a single set of four readings for change of pH as recorded by Bunzel (1915). Its action was tested only on hydroquinone. The “unnaturalness” of the conditions is attested by the fact that there is no absorption of oxygen in neutral solutions. Therefore no conclusions can be reached as to the relation of laccase to the phosphate ion in living cells in which the substrate is quite different and the enzyme is effective at the pH of protoplasm. Actually, the graph on the logarithmic plot was a straight line with slope -7.46 for three of the four readings, the fourth one being nearest the point of no oxidation.

The most complete data to be found for enzyme action in relation to phosphate are those for pancreatic lipase. In all, the results of five independent sets of experiments have been found to be available for analysis. The most recent workers (Platt and Dawson, 1925) recognized a specific function of the phosphate buffers which were also used by the previous workers. Their technique was likewise more refined and their data cover both change of pH and change of concentration. Hence, their results carry more weight and are considered first.

Platt and Dawson estimated the action of pancreatic lipase of the pig by titrating the butyric acid released through the hydrolysis of ethyl butyrate. They were concerned partly with the optimum pH and found it to be about 7.0 for phosphate buffers and purified lipase. By using glycerin extracts of the pancreas they were able to carry the pH as high as 8.0 with steadily increasing hydrolysis. They consider that a protection of the enzyme is afforded by some constituent of the glycerin extract. These experiments (Nos. 5 and 6) are therefore the ones from which we have obtained data for a wide change of pH with constant concentration of phosphate salt. They are shown in Fig. 5 from which it is evident that their Experiment 5, in which only 5 cc. of phosphate solution was used, gives an unbroken straight line through a wide range of $p\text{PO}_4$. Experiment 6 involved the use of 25 cc. of phosphate solution and the points (shown by \circ) are somewhat irregularly distributed. In both experiments the points represent single readings and additional experiments would probably remove the irregularities. The general trend of the points is not far from that for the lower concentration of salt the equation for which is

$$(\text{Hydrolysis}) (p\text{PO}_4)^{1.01} = K$$

After a specific effect of phosphate was observed the point was carefully studied by using different molar concentrations of the buffer salts (*no hydrolysis could be carried in the absence of phosphate*). In the first of these studies (their Experiment 9) low concentrations were employed at $p\text{PO}_4$ 4.9 (pH 7.6). The results show that for the range 0.005 to 0.05 M there is a hyperbolic relationship between the

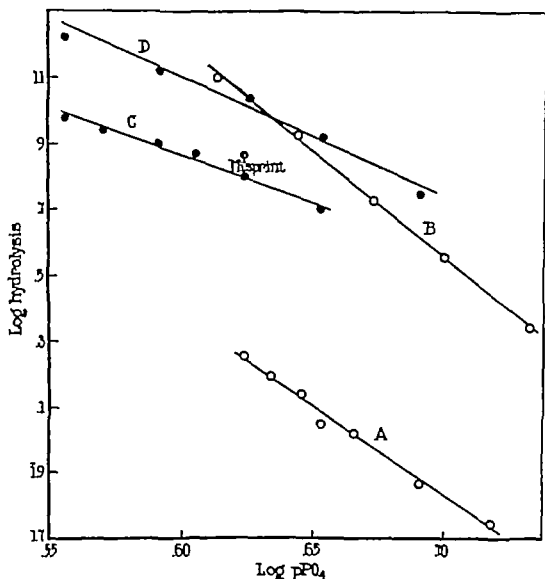


FIG. 6 Logarithmic plot of the relation of pPO_4 to the rate of hydrolysis of ethyl butyrate by pancreatic lipase. Data are taken from the work of Platt and Dawson (1925) in which the pPO_4 was varied by the use of varying concentrations of phosphate salts at constant pH. Each point represents the mean value of two readings. The equations for the lines, together with the pH used for each, are as follows

Curve A (Hydrolysis)	$(pPO_4)^{0.64} = K$	pH = 7.6
B (Hydrolysis)	$(pPO_4)^{0.25} = K$	pH = 7.2
C (Hydrolysis)	$(pPO_4)^{1.0} = K$	pH = 7.6
D (Hydrolysis)	$(pPO_4)^{1.25} = K$	pH = 7.6

The misprint indicated in connection with Curve C was obviously a mistake in one figure of the number given in the original paper

amount of hydrolysis and the $p\text{PO}_4$. The logarithmic plot is given in Fig 6, Curve A, which indicates the relation

$$(\text{Hydrolysis}) (p\text{PO}_4)^{0.44} = K$$

The method of obtaining the $p\text{PO}_4$ for the various concentrations is the same as that used in similar conversions for pH and was done by the use of the table of factors given by Clark (1922^b) for this purpose. Thus the $p\text{PO}_4$ at 0.01 M (all

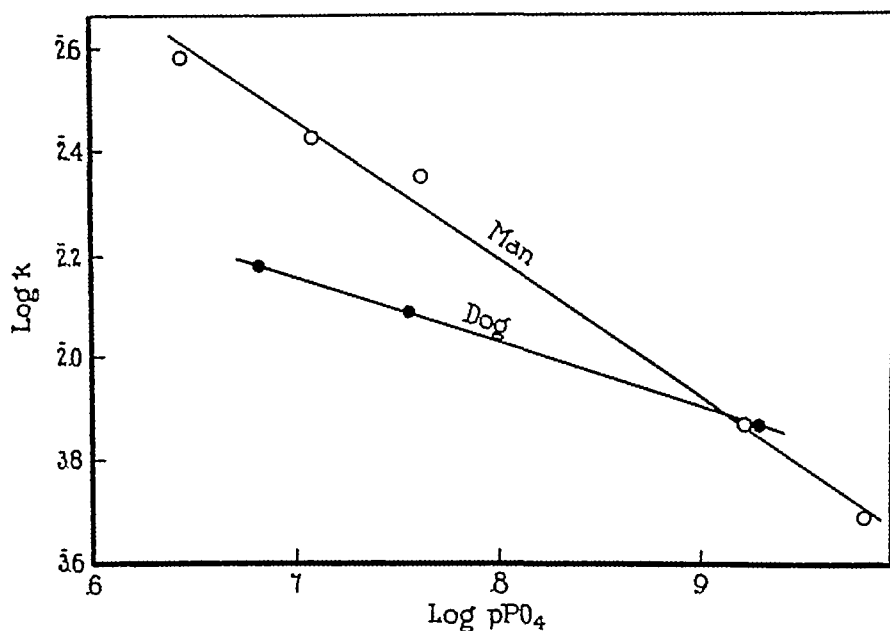


FIG 7 Logarithmic plot of the relation of $p\text{PO}_4$ to the action of pancreatic lipase on tributyrin. Data are taken from the work of Rona and Pavlović (1922). The $p\text{PO}_4$ was varied by using buffer solutions of pH 4.94 to 8.01. The equations for the lines are

$$\begin{aligned} \text{for lipase from dog } (\bullet) - K (p\text{PO}_4)^{1.26} &= K \\ \text{" " " " (O) - } K (p\text{PO}_4)^{2.7} &= K \end{aligned}$$

Each set of points is taken from a single experiment

calculations were corrected to this concentration) is 4.9 while that at 0.03 M is 3×4.9 or 0.3×3.9 which is $p\text{PO}_4 4.42$.

In Fig 6 there are also shown the results of the remainder of the tests with change of concentration at constant pH. For the experiment shown by Curve B the pH was 7.2. This is nearer the optimum than was the case for Curves C and D in which the pH was 7.6. Platt and Dawson comment on the difference in

form between these two types of curves as plotted using molar concentration and say that the farther the pH is above the optimum, the more pronounced the curvature. This is somewhat noticeable in Curves C and D when the data are converted to a logarithmic plot. However the conditions in the experiments were

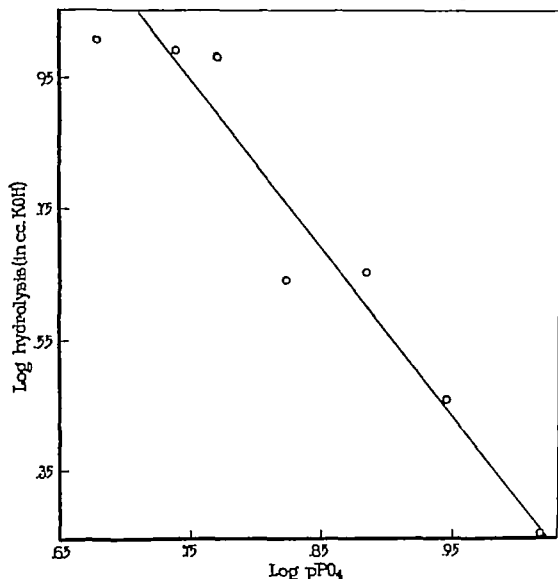


FIG. 8. Logarithmic plot of the relation of pPO_4 to the hydrolysis of olive oil by pancreatic lipase of the pig. Data are taken from a single experiment by Umeda (1915). The pPO_4 was varied by the use of buffer solutions of pH 4.494 to 7.731. The equation for the line as drawn is

$$(\text{Activity of enzyme}) (pPO_4)^{1.44} = K$$

essentially the same as for those shown by Curve A. (This was also our justification for suggesting the indicated misprint in the data used for Curve C see legend.)

The equations for the four experiments in which the pPO_4 was altered by change of molar concentration are given in the legend of Fig. 6. They differ from the

equation for the same relationship derived by change of pH only in the slope of the line, which is also indicated by the exponent of $p\text{PO}_4$ in the equation. This difference is directly attributable to the attendant differences in molar concentration and hydrogen ion concentration. The straight lines on the logarithmic plots attest the hyperbolic relationship between $p\text{PO}_4$ and activity of the lipase, *whether the concentration of the $\text{PO}_4^{'''}$ ion be increased by decrease of pH or by increase of total salt concentration*. The slope merely measures the sensitivity of the given sample of enzyme to the change in concentration of the $\text{PO}_4^{'''}$ ion under the experimental conditions imposed by other factors.

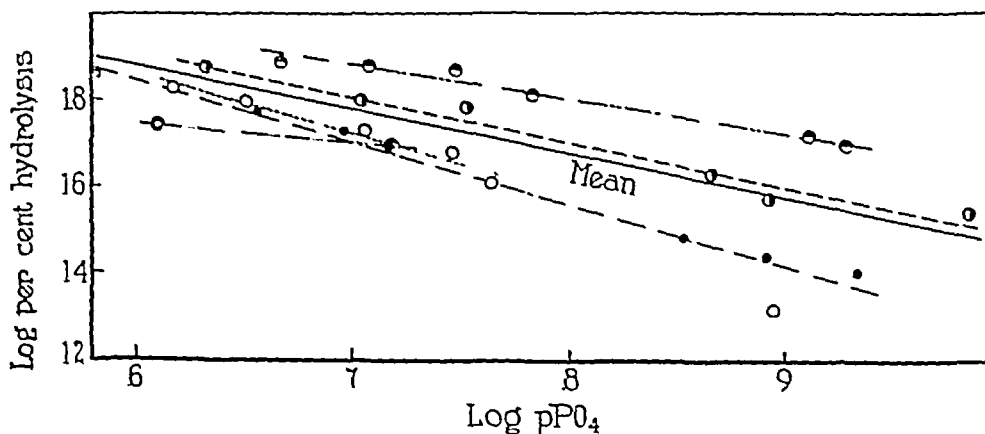


FIG. 9. A composite logarithmic plot of the relation of $p\text{PO}_4$ to the percentage hydrolysis of tributyrin by the pancreatic lipase from beef pancreas. Data are taken from five single experiments by Rona and Bien (1914), each of which is represented by a broken line. The equation for the mean line as drawn (solid line) is

$$(\text{Hydrolysis}) (p\text{PO}_4)^{1.08} = K$$

The $p\text{PO}_4$ was varied by the use of buffer solutions of pH 4.87 to 8.58.

Platt and Dawson also noticed that both α - and β -sodium glycerophosphate promoted the action of lipase to the same degree. They state their opinion that this indicates an effect by the phosphate ion. The present analysis confirms this observation.

Another recent measurement of the action of pancreatic lipases (purified) on tributyrin is reported by Rona and Pavlovic (1922). The available data are very meager but the uniformity of the results is evident from the form of the plots in Fig. 7. Only three measurements are available for the lipase from dog pancreas and there are but five for that from the human pancreas. Each set of points, however, lies along a straight line, the slope of which differs for the two types of enzymes. The V used as a measure of the activity of the enzyme was calculated by the authors from a monomolecular equation for the hydrolysis. In each case the $p\text{PO}_4$ was altered by change of pH.

The work of Umeda (1915) furnished a single set of readings of the hydrolysis of olive oil by purified pancreatic lipase of the pig in relation to pPO_4 as calculated from the pH. The lipolysis was estimated by titration of the acid produced after 20 hours. Fig 8 shows the same hyperbolic relationship to pPO_4 . Here the equation is

$$(\text{Activity of enzyme}) (pPO_4)^{1.44} = K.$$

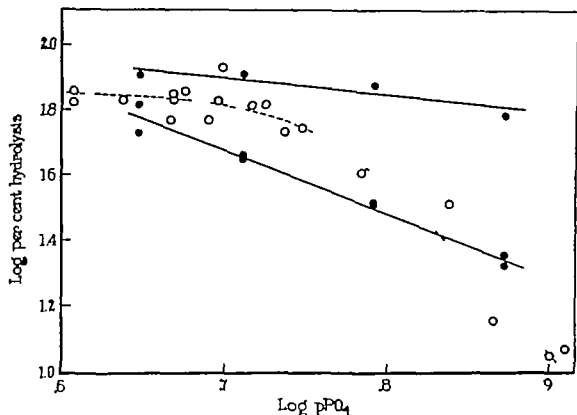


FIG 10 Logarithmic plot of the relation of pPO_4 to the percentage hydrolysis of fat by pancreatic lipase of the pig (shown by (●) and solid lines) and by lipase of the duodenal juice (shown by (○) and broken line). Data are taken from single readings by Davidsohn in 1913.

Rona and Bien (1914) used a glycerin extract of lipase from beef pancreas and estimated the hydrolysis of tributyrin by a stalagnometric method. This method is not so accurate as titration. The results are converted into percentage of hydrolysis and plotted against pPO_4 , as usual in Fig 9. This is a composite of several experiments, each of which is indicated by a broken line. The variation from the average line is due largely to the variation in the activity of the sample of enzyme used. Each experiment gave a straight line plot and the mean slope is -1.08 . Therefore the equation is the same as those derived from later measurements.

In Fig 10 are shown the results from a still earlier report (Davidsohn 1913) of hydrolysis of fat by pancreatic lipase of the pig. This is the only case we have

found in which all the points do not lie along a straight line on this type of plot. The two solid straight lines in the figure are drawn in respect to the results from three experiments with lipase direct from the pancreas, one sample of which was obviously more active than the other two. These data clearly exhibit the same type of relationship as has been demonstrated in the previous analyses.

The other points (\bar{O}) on this composite plot represent data from a number of single measurements with lipase contained in the duodenal juice. The curved, broken line roughly indicates the trend of these points as plotted over a wide range of pH and $p\text{PO}_4$. The points at the extremes could be accounted for on the basis of the unfavorable pH of the media. However, the distribution of all these points is not only very irregular but uncertain. This is due to the composite nature of the data, the use of the stalagmometric method of analysis, the variation in the total concentration of phosphate buffer salts, and the inability to correct for all such factors in converting to $p\text{PO}_4$, because of the incompleteness of the statement of experimental procedure. The relationships exhibited by our other analyses of lipase studies and the recent demonstration by Platt and Dixon of an absolute dependence of pancreatic lipase, also of the pig, upon the presence of at least some phosphate, appears to outweigh the doubtful evidence from the older results as regards the lipase obtained in a different medium.

From these analyses of the relation of lipase, peroxidase, and possibly laccase to phosphate solutions, it seems quite certain that the PO_4''' ion acts as a promoter of their activities. The mathematical statement of the relationship is like that for the effect of phosphate on the production of CO_2 by living cells. There is then every reason to believe that the active component of such phosphate solutions is the PO_4''' ion, acting as a promoter catalyst. The very fact that the mathematical expression of the relationship is of the form

$$(\text{Activity of enzyme}) (p\text{PO}_4)^n = K$$

is of itself an additional proof, for the term " $p\text{PO}_4$ " is a direct measure of the potential of the PO_4''' ion in a given solution. The inverse proportionality expressed by the equation is really a direct proportion because of the peculiar method of statement of the potential.

In the case of plant respiration the exponent of the $p\text{PO}_4$ term was found to be 1. The corresponding exponent in the case of peroxidase was essentially the same (1.34). Although an exponent of this order was also found for a few cases in the lipase analyses (*cf* Figs. 5, 7, and 9), the value 3 or 6 was more characteristic of lipase (*cf* Figs. 6 to 8). The agreement of the numbers of the "oxidase" group of enzymes is significant while the question of sensitivity of lipase (measured by

the value of the exponent, n) to the phosphate ion is beyond the immediate scope of our problem

III.

SUMMARY

The active component of phosphate solutions, in relation to promoter action on oxidising enzymes, is the PO_4''' ion. This is shown by the demonstration of a hyperbolic relationship between per cent production of CO_2 (of *Elodea*) and pPO_4 , the measure of the phosphate ion potential. This is consistent with the rate of respiration as affected by changing pPO_4 through change of total phosphate concentration while pH is kept constant. The equation for this relationship is

$$(\text{CO}_2 - a) (\text{pPO}_4 - b) = K$$

where a , b , n , and K are constants and $n = 1$

The same relationship to phosphate ion concentration, expressed by the equation

$$(\text{Activity of enzyme}) (\text{pPO}_4)^n = K,$$

where n and K are constants and n varies from 1 to 6 under different conditions, appears to hold for some other enzyme actions, including those of peroxidase and pancreatic lipase.

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ARSENATE AS A CATALYST OF OXIDATION

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In connection with another paper describing the catalytic rôle of phosphate in plant respiration (Lyon, 1927) brief mention is made of some experiments in which the effect of arsenate on plant respiration and on oxidation was observed qualitatively. A catalytic effect is found in both cases. The importance of this hitherto unconsidered property of arsenate seems to justify a more detailed report of experiments which have been extended in order to provide more data.

It should be borne in mind that the present discussion of the catalytic effect of arsenate does not detract from the apparently unique rôle of phosphate as a non toxic promoter catalyst for the respiratory enzymes. The same concentration of arsenate which momentarily increases the rate of production of CO_2 by living tissues soon exercises a toxic effect.

There are several suggestive items of evidence to be found in the literature regarding the effect of arsenate salts. Most of those to which we shall refer have resulted from comparisons of arsenate with phosphate in models or isolated phases of respiration. These are therefore particularly valuable items of evidence.

In the oxidation reduction system of Haehn and Pölz (1924) a supposed cleavage of water molecules is effected by means of a combination of amino acid and phosphate. At least the presence of these two reagents in aqueous solution results in an oxidation of acetaldehyde and a reduction of methylene blue, at relatively high temperatures. Arsenate was found to be equally effective when substituted for the phosphate.

Meyerhof and Matsuoaka (1924) have repeated and extended the observations of Warburg and Yabusoe (1924) on the oxidation of fructose by pure oxygen in a solution of phosphates presumably containing iron salts. They found that arsenates could be used in place of phosphates without destroying the effectiveness of the iron catalysis.

The substitution of arsenate for phosphate in alcoholic fermentation cannot be made to the extent of a complete replacement of phosphate. Some phosphate is

essential to the formation of hexose phosphate, an ester of hexose and potassium phosphate. This ester is continually being formed and decomposed by certain enzymes. Harden and Young (1911) did observe that arsenate could replace phosphate in the sense that the latter causes an increase in the production of CO (and alcohol). They attributed this effect to an increased activity of hexose phosphatase, the enzyme which splits the ester.

When Harden and Henley (1922) reviewed the work of Witzemann (1920) on the oxidation of glucose by H_2O_2 as affected by phosphate, they were inclined to the view that the buffered solution of phosphates aided the reaction by the effect on the peroxide. They demonstrated a qualitatively similar action by other salts such as carbonates. However, their results show a special effect of phosphate and arsenates over and above the buffer action. This effect has never been clearly explained.

Our first experiments with arsenates consisted in adding them to slowly oxidizing solutions of pyrogallol and to slowly respiring tissues of *Elodea canadensis*.

Whenever a few drops of arsenate solution—either the alkaline (pH = 9.18) solution of disodium arsenate or the same solution brought to pH 7.0 with the same molar concentration of arsenic acid—are added to a relatively large volume of a solution of pyrogallol, there is a rapid coloration of the solution. This indicates an oxidation of the pyrogallol, the end-products being purpurogallin, carbon dioxide, and water. It is a matter of hours before the usual slow oxidation forms sufficient purpurogallin to give a pale straw color to the solution, but the addition of arsenate provides the same color in not more than 2 minutes. The effect of arsenate is qualitatively the same as that of phosphate (*cf.* Lyon, 1927).

In a typical experiment 5 drops of a 0.085 M solution of disodium arsenate are added to 15 or 20 cc. of 1 per cent solution of pyrogallol. For the control there is added the same number of drops of a solution of NaOH which has been diluted until the hydroxyl ion concentration is the same or greater than that of the arsenate solution. The difference in color between the experiment and control increases rapidly during the 1st hour or 2 and is more marked near the surface of the solution. The difference is maintained for days and weeks while both solutions become yellowish, then brown, and finally brownish-purple. No previous mention of such an effect has been found.

In Fig. 1 are shown six individual time curves of the rate of production of CO_2 by *Elodea canadensis*. These experiments were carried out

according to the technique described in a previous paper (Lyon, 1923-24), using a form of the apparatus described by Osterhout (1918-19). The normal rate of production of CO_2 by the untreated tissues is taken as 100 per cent. The subsequent changes in the rate are indicated by a curve smoothed through points each of which represents the rate measured at a certain time after the addition of a sufficient volume of

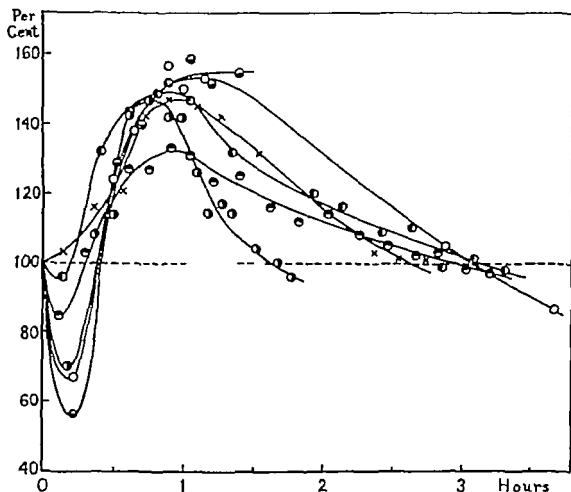


FIG. 1. The effect of 0.1 M neutral arsenate solutions on the production of CO_2 by *Elodea*. Each curve represents a single experiment. The normal rate before the addition of arsenate is taken as 100 per cent.

a concentrated solution of neutral mixtures of disodium arsenate and arsenic acid to give an effective concentration of 0.1 M.

The fall of the first readings below normal is to be regarded as the result of the increased CO_2 -absorptive capacity of the diluted solutions of arsenates (cf Fig. 1 in Lyon, 1923-24). The rise of each curve above the 100 per cent level shows the manner in which the rate of

production of CO_2 is uniformly increased after about 1 hour. The similarity of this part of each curve with that of the individual curves for the effect of phosphate (*loc cit*) is evident. This close parallel may lead us to believe that arsenate also acts as a catalyst to the respiratory enzymes of *Elodea*.

The difference in the effects of arsenate and phosphate is shown by the difference in their time curves after the 1st hour. With arsenate the toxic effect is shown by the gradual decrease in the rate of production of CO_2 until the rate becomes slower than the normal. With phosphate no such toxic effect is observed. The differences to be noted among the several individual experiments are to be attributed to the different rates at which the several lots of plants showed susceptibility to the toxic effect of the arsenate.

Until this apparent catalytic effect of arsenate has been demonstrated in a greater number of cases we may not go far in using the principle in an interpretation of the general action in organisms. We would, however, point out a possible relation of this catalysis to three of the four cases cited to show how phosphate may be replaced by arsenate. In the system used by Haehn and Pulz, in that of Meyerhof and Matsuoka, or in that of Harden and Henley, the substitution of arsenate for phosphate may be possible because each acts as a catalyst in the system. For example, in the case of the oxidation of fructose by pure oxygen in the presence of phosphate or arsenate and of iron, the phosphate or arsenate may act as a promoter catalyst to the slow or potential oxidation of fructose by iron.

To test the principle involved in this hypothesis we have performed, with arsenate, experiments comparable with those reported elsewhere for phosphate (Lyon, 1927). Into each of four tubes were placed 15 cc of a 1 per cent solution of pyrogallol. Additions of substances selected for an analysis of their effect on the rate of oxidation were made as follows: (1) 4 drops of 0.34 M neutral arsenate mixture, (2) the same plus a small, clean, iron nail, (3) 4 drops of water plus a similar iron nail, (4) 4 drops of water. No. 4 was a control and the rate of oxidation was so slow that no important color change could be observed for hours. The arsenate in 1 induced a visible production of color after not more than 2 minutes. Essentially the same color appeared in 2 above the nail and even less color finally came in 3

above the nail than in 4. But at the surfaces of the iron there appeared strongly colored regions. The region in 2 was much greater in volume than that in 3 but was violet purple in contrast with the pure purple about the iron in 3.

During the remainder of such an experiment the tubes were frequently stirred in order to avoid gradients in the concentrations of oxygen and oxidation products throughout the solution, particularly in the vicinity of the iron. This procedure caused no change in the apparent color of the oxidation products, but after 2 to 4 hours it was observed that the density of the coloration in No. 2 was much greater than that in either 1 or 3. To determine whether this was anything more than the additive effect of the two catalysts, the color was compared with that of a mixture of equal parts of 1 and 3, care being taken to equalize the dilution effect of the mixture. The result of such comparisons showed that the color in 2 was of a density greater than the purely additive effect would account for. It thus fully supported the hypothesis that arsenate can function as a catalyst to an iron catalysis—the so called "promoter effect."

These experiments have been repeated many times with uniform results, and likewise with pure iron wire to show that the iron in the nail alone catalyzed the oxidation. Attempts to obtain time curves for these experiments have been prevented by inherent difficulties. It is possible to find a suitable color standard for either the coloration produced by the arsenate and iron acting together, or for that of the color of the mixtures of solutions in which each acts alone, but the same standard will not do for both. This is due to an excess of pure purple produced when they act together and which is of itself a demonstration that the arsenate influences the action of the iron as a catalyst. By definition this is a promoter effect and corresponds to the observed effect on the respiratory enzymes of *Elodea* up to the time when it was masked by the toxic effect.

SUMMARY

Arsenate exerts a catalytic effect on the oxidation of pyrogallol by atmospheric oxygen, on the catalytic oxidation of pyrogallol by metallic iron, and on the presumably enzymatic production of CO₂ by *Elodea canadensis*.

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A THEORETICAL CONSIDERATION OF THE ACTION OF X RAYS ON THE PROTOZOAN COLPIDIUM COLPODA.

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I. INTRODUCTION

To show that the variation in reaction among the individuals of a group of apparently similar organisms fits a probability curve is, of course, not an end in itself, but rather a basis of conjecture as to the mechanism which provides the random element without which the theory of probability is meaningless. It is generally assumed that the reactions of various individuals differ because the individuals themselves are different. It is interesting, however, to consider the possibility that in some cases, where the organism is relatively simple, the individuals are essentially similar and the random element is inherent in the agent or in its primary effects. For example, if the agent is radiation, the quite generally accepted theory of absorption in *quanta* provides the necessary random element, and it may be that this is sufficient to account for the variations which we observe in the reaction to the rays of apparently similar simple organisms.

In his recent work on the action of x rays on *Colpidium colpoda*, Crowther¹ finds that if he plots the number of survivors against the dose of radiation administered the result is a sigmoid curve, and, assuming the animals to be essentially alike, he shows that this is the kind of curve to be expected if the animal dies as a result of a certain number, n , of discreet events or "hits," all equally effective, and if the probability per unit dose of making a hit is constant. He finds, further, that theory agrees quantitatively with experiment when λ , the probability per unit of dose, is 5.9×10^{-4} , and n (for immediate death) is 49.

To account for this very small value of λ , he makes use of an inter-

¹ Crowther, J. A., *Proc. Roy. Soc. London, Series B*, 1926, c, 390.

esting hypothesis, which he advanced some time ago² for a similar purpose, that the effect is confined to some very small body or structure inside the animal. In doing so, he introduces certain difficulties, in my opinion. No general objection is raised against this hypothesis, which we may refer to as the "small-body theory." In this particular case, however, it appears to be inconsistent with the phenomenon which it was devised to explain, unless it is supplemented by certain rather novel assumptions as to the nature of the destructive effect of radiation on tissue. The nature of these assumptions will be brought out in what follows. An alternative explanation of the small value of λ , which does not involve the small-body theory will also be suggested.

II Primary and Secondary Effects of X-Rays

The primary effect of x-rays on the light atoms of which living matter chiefly consists is the ejection of a high speed electron from some of them chosen at random in both space and time. Each of these primary electrons ionizes a large number of other atoms by collision before it comes to rest. This secondary ionization appears to be the only effect which we need consider. There is no good reason for supposing that the atom from which a primary electron has been ejected is the seat of any considerable part of the destructive effect, the disintegration of the particular molecule which contains this atom can scarcely be thought of as being more important, in general, than that of any other molecule. There is, on the other hand, plenty of evidence in favor of the view that the destructive effect is associated with the ionization produced by the high speed electron, in comparison with which the ionization by direct absorption of the rays is negligible. In very simple photochemical systems, the reactions produced by x-rays or by α - or β -rays proceed at a rate which is directly proportional to the rate of ionization, it would be rash, of course, to assert that this is true in the case of tissue destruction.

If the x-rays are monochromatic, the primary electrons are all ejected with the same speed and energy, they travel approximately equal distances before stopping, and they knock off about the same

² Crowther, J. A., *Proc. Roy. Soc. London, Series B*, 1924, xcvi, 207.

number of secondary electrons. The various quantum events or units are, therefore, much alike. Likewise, the events which consist in the production of the various secondary electrons are alike in one respect at least—all of the electrons have the same properties.

Doubtless one or the other of these units corresponds to the hit mentioned above. In what follows, I shall refer to the release of a secondary electron as an *electron-hit*, and to the emission of a primary electron with its attendant phenomena as a *quantum-hit*. The units of destructive effect dealt with in Crowther's analysis will be called *effective hits*.

III Some Quantitative Estimates

Crowther used the K radiation of molybdenum, the α lines of which have a mean frequency of 4.23×10^{18} per second. The primary electron is thus ejected with an amount of energy, $h\nu$, equal to 2.76×10^{-8} ergs. Dividing this by 5.5×10^{-12} ergs, the work required, on the average, to release a secondary electron in air according to Rutherford,² we find that each primary electron releases about 500 secondary electrons. This number will be denoted by E_1 .

The fourth power law, together with Whiddington's⁴ constant for air, shows that the maximum path length of the primary electron in air must be about .2 cm. The value taken directly from one of Sadler's⁵ curves is .22 cm. Evidently the law holds nicely even for these very soft rays. In tissue, assumed equivalent to air of unit density, the maximum path, L , is, therefore, about 2.6×10^{-4} cm.

The paths of the primary electrons are, in general, not straight, and, in consequence, the distance in a straight line from the beginning to the end of the path is generally less than L . Consider a plane layer of air, the thickness of which, x , is uniform and somewhat less than L . If a great number of electrons enter this layer through one of its faces, all with the same speed, but in all possible directions, some of them will emerge from the opposite face with some part of their original energy. A fraction, then, of the energy which goes into the layer on one side comes out on the other side. Sadler⁵ has shown that

² Rutherford, E., *Radioactive substances and their radiations*, Cambridge 1913 159

⁴ Whiddington, R., *Proc. Roy. Soc. London Series A* 1911-12 lxxxvi 360

⁵ Sadler C. A., *Phil. Mag. Series 6* 1910 xix, 337

mately of unit density, an e dose corresponds to the production of 1.63×10^{12} secondary electrons per cc. of tissue. This number will be called E .

IV The Small Body Theory

That λ_e has been found to be very small shows that very few of the hits received by the animal, whether electron hits or quantum hits, are effective. From the fact that the atoms from which the high speed electrons are ejected are distributed at random in space, it follows that the probability that a high speed electron will be ejected from within any small portion of the animal is directly proportional to the volume of the portion considered and independent of its position. The same is true of the probability that a secondary electron will be released within the portion considered, provided, of course, that the volume is such that the electron hits occur independently of one another. Assuming that the destructive effects are confined to some small body within the animal, we may assign volumes to this body such that either of these probabilities will assume any desired value,—in particular the value 5.9×10^{-4} in which case every hit within the small body will be effective. On the hypothesis that the electron hit corresponds to the unit of destructive effect, the diameter of the body (assumed approximately spherical) must be about 8.8×10^{-4} cm. Similarly on the quantum hit hypothesis, the diameter is about 7.0×10^{-4} cm.⁷

Let us now inquire whether this theory is consistent with the postulates on which the statistical treatment of the problem is based. The postulates are (1) that all effective hits are equally effective, and (2) that λ is constant.

Let us consider first the bearing of the electron hit hypothesis on the small body theory. L , the path length of the primary electron, is 29.4 times the diameter of the small body appropriate to the hypothesis that the individual secondary electron corresponds to a unit of destructive effect. Since 500 such electrons are released by the primary electron in traveling a distance equal to L , it is evident that

⁷ As the result of an error in calculation which Dr. Crowther discovered after publication, the diameters assigned to the body in his paper differ somewhat from those given above.

in most cases, in which a high speed electron traverses the small body, it will release more than one secondary electron inside it. Those events, then, which are at random in time, are not the electron-hits at all, but rather showers of electron-hits, and the number of electrons per shower must vary within very wide limits, because the intensities of ionization, at the beginning and the end of the path, respectively, differ so much, and because the length of the path through the body varies from zero to the length of the diameter,—even more if the path is not straight. The average number of electrons per shower is, of course, very great in the case of primary electrons which enter from outside and come to rest inside the body, and correspondingly small for those which are ejected from within the body. For primary electrons which pass through the body, the average number of secondaries per shower is about 11, since the mean length of a great number of straight paths through a sphere, chosen at random, is equal to two-thirds of the diameter. Electron-hits are not then at random in time—not even approximately so—and postulate 2 is not fulfilled.

It is apparent then that we must abandon either the electron-hit idea or the small-body theory.

We have now to deal with the quantum-hit hypothesis in its relation to the small-body theory. Let us assume for the sake of the argument that the distribution of the destructive effect along the path of the primary electron is the same as that of the secondary ionization—which would be true if we were dealing with a simple photochemical system. It is evident that some of the primary electrons, ejected from atoms inside the small-body, must escape from the body with a considerable part of their initial energies. Likewise, other high speed electrons, ejected from matter outside the small-body, will enter it before coming to rest. In these cases, the effectiveness of the hit will be less than in the cases in which the whole path lies inside the body. Hits of this kind will be referred to in what follows as “partial” hits.

The relative number of partial hits cannot be so small as to be negligible. On the quantum-hit hypothesis, the diameter of the small-body is 7.0×10^{-5} cm, whereas L is 2.6×10^{-4} cm, i.e. 3.7 times the diameter. In order to make a very rough estimate of the relative importance of the partial hits, we set λ equal to 3.5×10^{-5} cm, the

radius of the sphere, in the expression $e^{-\alpha r}$ discussed in Section III, and we find that, of the energy associated with high speed electrons ejected from points midway between the faces of a layer of tissue of thickness equal to the diameter of the small body, 66 per cent escapes from the layer. If we say that 66 per cent of the electrons escape, we shall make an underestimate, for each of the escaping electrons has lost a part of its initial energy. If we say that 66 per cent of the high speed electrons, ejected from the center of the small body, escape we shall underestimate the number still further, because the radius of the sphere is much smaller than the mean of the distances between a point in the middle of the plane layer and the points where the electrons escape from the surfaces of the layer.

Of the high speed electrons ejected from the center of the small body, then, at least 66 per cent escape, of those ejected from points near the surface of the body, at least 50 per cent escape. Let us say that at least 50 per cent of all high speed electrons released within the small body escape from it. Now for every one which escapes, another enters from outside. The whole number of hits, both total and partial, is the whole number of those events which occur at random in time is then increased by 50 per cent and two-thirds of them are partial hits. To keep the whole number down to 49, the body must be made smaller, and this will make the relative number of partial hits still greater. From what has been said in Section III about the distribution of ionization along the path it appears that we must give up either the small body theory or the idea *that the distribution of the destructive effect along the path of the high speed electron is similar to that of the ionization*.

It is conceivable that the destructive effect, though brought about by ionization, is not measured by it, that it is conditioned in some way by the density of ionization or otherwise. It might be supposed, for example, that at the end of a path a small portion of tissue is injured so seriously that repairs are impossible, that at other points along the path the injury, being diffuse, is rapidly made good. If this were true and if the permanent injury which corresponds to an effective hit were confined to a very small region—to 1 per cent, let us say, of the path length, no objection could be raised against the small-body theory.

certain particular electrons. The problem cannot be analyzed, of course, too little is known about the fine structure of matter. In what follows, an attempt will be made to estimate the various quantities involved in the relatively simple case where Y is formed when λ , represented by an idealized molecule, loses one particular electron. It will be assumed that in a microscopic sense the molecules of λ are at all times distributed at random in space insofar as the finite size of the molecule permits,—the arrangement to be expected in a solution. It will appear further on that the molecule would have to be extremely large to have an appreciable effect on this distribution, it will be assumed tentatively that it has none.

Multiple Effective Hits Made by One Quantum

Let us assume for the moment that, in the ordinary sense, Y is distributed uniformly throughout the whole volume of the animal. If the probability that a quantum, falling entirely inside the animal, will make an effective hit be represented by p , and if V be the volume of the animal, then

$$p = \frac{\lambda_e E_q}{E_\lambda V} \quad (1)$$

V may be taken as 10^{-7} cc. Using the value of λ_e given by Crowther, and the values of E_q and E_λ found in Section III, we find that $p = 1.8 \times 10^{-4}$. Now it is not the quantum as a whole, but rather the individual secondary electrons which correspond to the hits. In the language of probability we may, therefore, speak of the number of "trials" per quantum. If the molecule of Y were so very small that it would never lose two or more electrons, the number of trials would be equal to E_q . If the molecule were larger, the number of trials would be less than E_q . When p is less than 1, a decrease in the number of trials, corresponds to a decrease in the ratio of p to p_1 , p being the probability that the quantum will make exactly r effective hits and p_1 the probability of exactly one such hit. For example, if the number of trials were 1, the probability of a multiple hit would be absolutely zero. To find the maximum value of this ratio, which we may call R_r , we take the number of trials as infinite in which case R_r is $p^{r-1}/r!$. In particular R_2 is $p/2$ or 9.0×10^{-7} .

R_r is so small that we may now reconsider the assumption that X is distributed uniformly throughout the whole volume of the animal. Other things being the same, p is proportional to the number of molecules of X per unit volume in the region where the quantum falls. If in some part of the animal the concentration of X were 1000 times as great as the mean concentration, then in this part p would be 1.8×10^{-3} , only about one effective hit in a million would be a "double," and one in less than 10^{12} a "triple" hit. p , for a particular quantum, cannot, of course, be greater than the value corresponding to the maximum concentration which the primary electron encounters, the concentration may change from point to point, therefore, as abruptly as desired.

It is evident, then, that effective hits are at random in time and that the molecules of X to be hit effectively are chosen at random, even though no unreasonable restrictions are placed on the way in which X is distributed.

The Size of the Molecule

We have now to deal with the slow change in λ , which takes place in consequence of the fact that N is finite. Let P_1 be the probability that a destroyed molecule of X , chosen at random from among the whole number of those that have been hit, will have lost exactly one electron, and let P' be the probability that a molecule, chosen at random from among all those which have lost exactly one electron, will have lost the particular electron required. In the normal case, the making of n effective hits corresponds to the destruction of n/P_1P' molecules of X , and, therefore, n/NP_1P' represents the relative change in λ . This latter quantity must then be small, just how small is a matter of judgment. It ought certainly to be smaller than the errors in experiment, and the results of Crowther's experiment fit the theoretical curve very nicely. The values of P_1 and P' depend on the properties of the molecule of X . As the volume, v , and the complexity of the molecule increase, both P_1 and P' diminish, furthermore, since Nv may not be greater than the whole volume of the animal, the maximum value which we may assign to N diminishes. The hypothesis is, therefore, consistent for a given value of v provided the corresponding value of NP_1P' is sufficiently large in comparison with

n and provided the value of P_1P' appropriate to a molecule of volume v is not so small that N has to be greater than V/v . It is obvious that these conditions are more easily fulfilled the smaller and simpler the molecule. We have to find out, if possible, whether or not they are fulfilled when the molecule is fairly large.

In order to estimate P_1 and P' , it is necessary to make certain idealizing assumptions as to the nature of the molecule and to assign a definite size to it. To make it possible to treat P_1 statistically, it is assumed that the molecule will behave as though its electrons were distributed at random inside a sphere, the volume of which is the same as that of the molecule, the probability of releasing an electron being the same for all of them. For convenience, the diameter of the sphere is set equal to 10^{-7} cm. The volume is then equal to that of the molecule of oleic acid according to Langmuir.*

In the case of a complex organic molecule the electrons must be fairly evenly distributed throughout what we call its volume, ϵ the room which it occupies when stacked with other molecules to constitute matter in the solid state. Such a distribution, together with the movements of the electrons, and the random orientation of the molecule with respect to the path of the high speed electron may reasonably be thought of as equivalent to a random distribution. The probability that an electron will be released from the molecule is then directly proportional to the path length through the molecule. The constant of proportionality will be nearly enough equal to that for tissue in general, if we assign to the molecule the same number of electrons as that in the molecule of oleic acid, $\epsilon = 158$. P' will then be 0.063. It will appear presently that, for a molecule of this size, the conditions imposed by the size of the animal and the desired constancy of λ , are fulfilled with a margin of safety which is so great that the errors involved in idealizing the molecule need not be small.

We must now try to estimate P_1 . Since the particular electron to be removed may be anywhere, we must suppose that it is in the worst place ϵ at the center of the sphere. It will have the same chance of being hit wherever it is, but if it is at the center, the primary electron must traverse the longest path through the sphere to reach it,

* Langmuir, I., *J. Am. Chem. Soc.* 1917 **xxxix**, 1848.

and the probability of removing two or more electrons from the same molecule increases with the path length. It has been shown in Section III that, on the average, 30 per cent of the ionization, i.e. 150 secondary electrons, lie in the first half of the path of the high speed electron, and that even at the midpoint of the path, consecutive secondary electrons are no closer together on the average than 7.3×10^{-7} cm, which is over seven times the maximum path length through our molecule. Let us confine our attention to the first half of the path for the moment. If the high speed electron were shot into a solid mass of *X*, the molecules being lined up in such a way that it would traverse a diameter of each, only about one molecule in seven at the midpoint of the path would lose an electron. When we remember that electrons are released farther and farther apart as we go from the midpoint toward the beginning of the path, we see that the number of cases in which a molecule loses two or more electrons must be very small in comparison with the number of those in which it loses only one. We shall make no great error if we assume that all hits in the first half of the path are "single hits." There are, undoubtedly, many single hits in the last half of the path, where the ionization is more intense, but we shall ignore them in order to make sure that we are not over-estimating P_1 . The total number of single hits is then equal to 150, the number of electrons in the first half. It should be remembered that we assumed that the path follows the diameter of the molecule for the purpose of estimating the relative number of double hits. The number of single hits just found, 150, has nothing to do with the exact location of the path.

Now P_1 is, in the long run, the ratio of the number of molecules which have lost one electron to the total number destroyed by the loss of any number of electrons. For the average quantum, falling in a mass of *X* in the pure state, the whole number of molecules destroyed, which we will denote by M , must be less than 500, for some of the molecules lose two or more electrons. If all of the electrons in the last half of the path were lost by the same molecule, an absurd assumption, M would be 151, and P_1 would be 1. If all of the hits in the second half of the path were doubles, M would be 325 and P_1 would be 46. This is the minimum value of P_1 . To sum up— P_1 lies somewhere between 46 and 1, and M lies between 150 and 500. Even

though the value of P_1 depends in part on M , we must consider the limiting values separately

λ_e , the probability per e unit of making an effective hit, is given by

$$\lambda_e = \frac{E_e}{E_q} \lambda \cdot M P_1 P' \quad (2)$$

Substituting the limiting values of M , found above, we see that NP_1P' must lie between 6.9×10^4 and 2.3×10^4 . To be on the safe side, we use the smaller of these numbers to test the constancy of λ_e . $\lambda_e \div NP_1P' = 7.1 \times 10^{-4}$. In the normal case, λ_e may change, then, by as much as .007 of 1 per cent. Such a change is too small to consider.

Now we consider the maximum value of N . To make N as large as possible, we divide the greater value of NP_1P' , which is 2.3×10^4 , by the minimum value of P_1P' , which is $.46 \times .63 \times 10^{-4}$, and N comes out to be 8.0×10^8 . The total volume of X in the animal is then 4.2×10^{-12} cc., which is only 4.2×10^{-4} times the volume of the animal. The "volume" concentration of X is then only .0004 of 1 per cent, it is, of course, so small that cases will be very rare in which the finite size of the molecule interferes with the assumed random distribution.

The margins of safety in the variation of λ_e and in the total volume of substance X are obviously so great that the error involved in assuming that the real molecule behaves like the ideal one may also be very great without rendering the general hypothesis untenable. There can be little doubt that, if an effective hit corresponds to the removal of a particular electron from a molecule of X , the molecule of X may be fairly large and complex.

VI. CONCLUSION

If we accept the idea that the reactions of living matter to x rays are the result of ionization, we find that Crowther's small body theory serves to explain the small value of λ only provided it be assumed further that the unit of destructive effect which corresponds to an effective hit is associated with the quantum and that it is localized in a region the dimensions of which are very small in comparison with the path length of the high speed electron.

It is suggested that there exists in the animal a substance, X , distributed throughout a considerable part of the tissue, a molecule of which turns into a molecule of a substance Y when it loses a particular electron, and that the formation of a molecule of substance Y constitutes an effective hit. This hypothesis seems to be consistent if the molecule of X is not too large. There is, of course, no good reason for supposing that it is the true explanation of the phenomenon. It is put forward merely to show that we may accept the theory that the variations in reaction are inherent in the x-ray itself without accepting the small-body theory.

VII. SUMMARY

1 The theory which Crowther has advanced to account for the variation of the lethal dose of roentgen rays among the individuals of a group of *Colpidium colpoda* is reviewed.

2 It is shown that the use of his small-body theory to explain the small value of λ , leads to certain further assumptions about the nature of the destructive effect.

3 An alternative hypothesis is discussed.

TIME RELATIONS OF GROWTH

III GROWTH CONSTANTS DURING THE SELF ACCELERATING PHASE OF GROWTH *

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I.

INTRODUCTION

The period of growth of multicellular organisms, and of populations of organisms, may be divided into two fairly distinct phases (1) a *self-accelerating phase* during which the time rate of growth increases with the increase in size of the organism or the population, and (2) a *self inhibiting phase* during which the time rate of growth decreases with the increase in size of the organism, or population. The question of mechanisms bringing about the general similarity in the course of growth of animals, plants, and populations, need not be gone into in this place except to note that the course of growth is in all these cases governed, directly or indirectly, by the same two primal forces (1) the force inherent in all organisms to reproduce at a constant percentage rate, and (2) the growth-inhibiting forces resulting from the finite nature of the universe in which the organisms find themselves.

The purpose of this series of papers is merely to present quantitative analyses of growth curves, with special reference to developing methods for computing *rational* growth constants. The first two papers¹ were concerned with the *self inhibiting phase* of growth, that is the phase of growth following the major inflection in the time curve.

* The principal portions of this paper have been presented before the Physiological Section of the Botanical Society of America, in Philadelphia, December 30 1926.

¹ Brody, S. *J. Gen. Physiol.*, 1925-27, viii 233. Brody, S. Sparrow, C. D., and Kibler, H. H. *J. Gen. Physiol.*, 1925-26 ix 285.

of growth This paper is concerned with the *self-accelerating phase* of growth

II

Proposed Methods for Computing Growth Rates

Four methods have been proposed for computing growth rates

1 *The Method of Minot*—Minot computed the rate of growth by dividing the gain in weight during a finite unit of time by the weight at the beginning of the unit of time, as represented by the equation

$$R = \frac{W_2 - W_1}{W_1} \quad (1)$$

or

$$W_2 - W_1 = R W_1 \quad (1a)$$

in which W_1 and W_2 are, respectively, the weights at the beginning and the end of the unit of time, R is the relative (or when multiplied by 100, the percentage) rate of growth

Employing this method he was led to the conclusion that, in warm blooded animals, the percentage rate of growth declines from 1000 per cent per day shortly after fertilization, to 3 to 7 per cent per day at the time of birth or hatching

There is this objection against the use of equation (1), for the self-accelerating phase of growth It is based on the assumption that growth is a discontinuous process, *i e*, that the increments are added at arbitrary time intervals, t_1, t_2, t_3 As a matter of fact, statistically considered, growth is a continuous process, and the relative rate of growth, must, therefore, be represented by the equation

$$k = \frac{dW/dt}{W} \quad (2)$$

or

$$\frac{dW}{dt} = kW \quad (2a)$$

in which k is the instantaneous relative rate (or when multiplied by 100, percentage rate) of growth, corresponding to R in equation (1)

The error introduced by the use of equation (1) is very considerable, as may be seen from the following considerations

From equation (2), at time t_1 , the weight, W_1 , of the organism is represented by the equation

$$W_1 = Ae^{kt_1} \quad (3)$$

At time t_2 , it is represented by

$$W_2 = Ae^{kt_2} \quad (3a)$$

Subtracting the former from the latter we obtain,

$$W_2 - W_1 = Ae^{k(t_2 - t_1)}$$

Dividing by (3),

$$\frac{W_2 - W_1}{W_1} = e^{k(t_2 - t_1)} - 1$$

Transposing and taking logarithms,

$$\ln \left(\frac{W_2 - W_1}{W_1} + 1 \right) = k(t_2 - t_1)$$

For 1 unit of time,

$$k = \ln \left(\frac{W_2 - W_1}{W_1} + 1 \right) = \ln(R + 1) \quad (4)$$

Numerical relations between k , the instantaneous rate of growth, and R , the rate of growth as determined by Minot, may be computed by substituting the values of R in equation (4). The results for a series of substitutions are presented graphically in Fig 1. Fig 1 makes it clear that Minot's method (equation (1)) can not be used for computing the relative rate of growth when the rate exceeds 10 per cent for the unit of time under consideration.

2 The Method of Pearl—In 1907

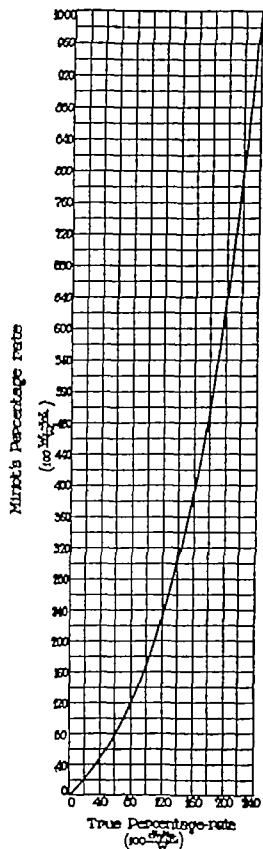


FIG 1 The relation between the increase in percentage rate as computed by the logarithmic method and by the arithmetical method of Minot

Pearl proposed the method for computing growth rates represented by the equation

$$\frac{dW}{dt} = \frac{k}{t - a} \quad (5)$$

which in the integrated form is,

$$W = A + k \ln (t - a) \quad (5a)$$

Equation (5) cannot, evidently, be used to represent growth during the self-accelerating phase of growth, inasmuch as the time rate of growth is represented by (5) to decline with time, while during the self-accelerating phase, growth increases with time

3 *The Method of Robertson*—In 1908 Robertson suggested that the equation

$$\frac{dW}{dt} = k W (A - W) \quad (6)$$

or

$$\frac{dW/dt}{W (A - W)} = k \quad (6a)$$

may be used to represent certain phases of growth termed by him *growth cycles*. Equation (6) indicates that the velocity of growth is a function not only of the size, W , of the organism, but also of growth yet to be made, $(A - W)$. The objections against this equation are indicated in the following sections

4 *The Method of Pearl and Reed*—Pearl and Reed introduced the following modification in the autocatalytic equation (6), employed by Robertson. They replaced k , by "some as yet undefined function of time," $F(t)$, "since the rate of growth of W is dependent upon factors that vary with time." They then assumed that $F(t)$ may be replaced by the series

$$k_1 t + k_2 t^2 + \dots + k_n t^n$$

thus changing equation (6) into

$$\frac{dW/dt}{W(A - W)} = F(t) = k_1 t + k_2 t^2 + k_3 t^3 + \dots + k_n t^n \quad (7)$$

They found the integrated form of equation (7) to be elastic enough to fit the growth curve of the rat beginning with 10 days after birth

Since, however, the period preceding 10 days after birth is an exceedingly important one, and since the constants in equation (7) do not have definite physical meaning (thus, when $(A-W) = 1$, $\frac{dW/dt}{W} = k_1t + k_2t^2 + \dots + k_nt^n$, what is the physical meaning of

k_1t, k_2t^2, \dots ?) therefore the method of Pearl and Reed is not suitable for the purpose under consideration (which is to evaluate *rational* growth constants, *i.e.*, constants having well defined physical meaning)

III

The Method Employed in This Paper

During the self-accelerating phase of growth, when the time rate of growth increases with the increase in the size of the organism, it is reasonable to attempt to relate the time rate of growth, $\frac{dW}{dt}$, to the size, W , of the organism, by the function

$$\frac{dW/dt}{W} = k \quad (2)$$

or

$$\frac{dW}{dt} = k W \quad (2a)$$

The first thought is that the constancy of k may be tested by integrating (2) and solving for k

$$k = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

As a matter of fact this is an impractical procedure for two reasons. First, it is not known for how long a period equation (2) represents the data, *i.e.*, $t_2 - t_1$ may represent more than one stage or cycle of growth, second, ratios are very sensitive to slight changes in one or both of the variables. This fact taken with the large experimental errors involved in this work, makes the results apparently erratic.

A better method is to plot the logarithms of the size, or weight, of

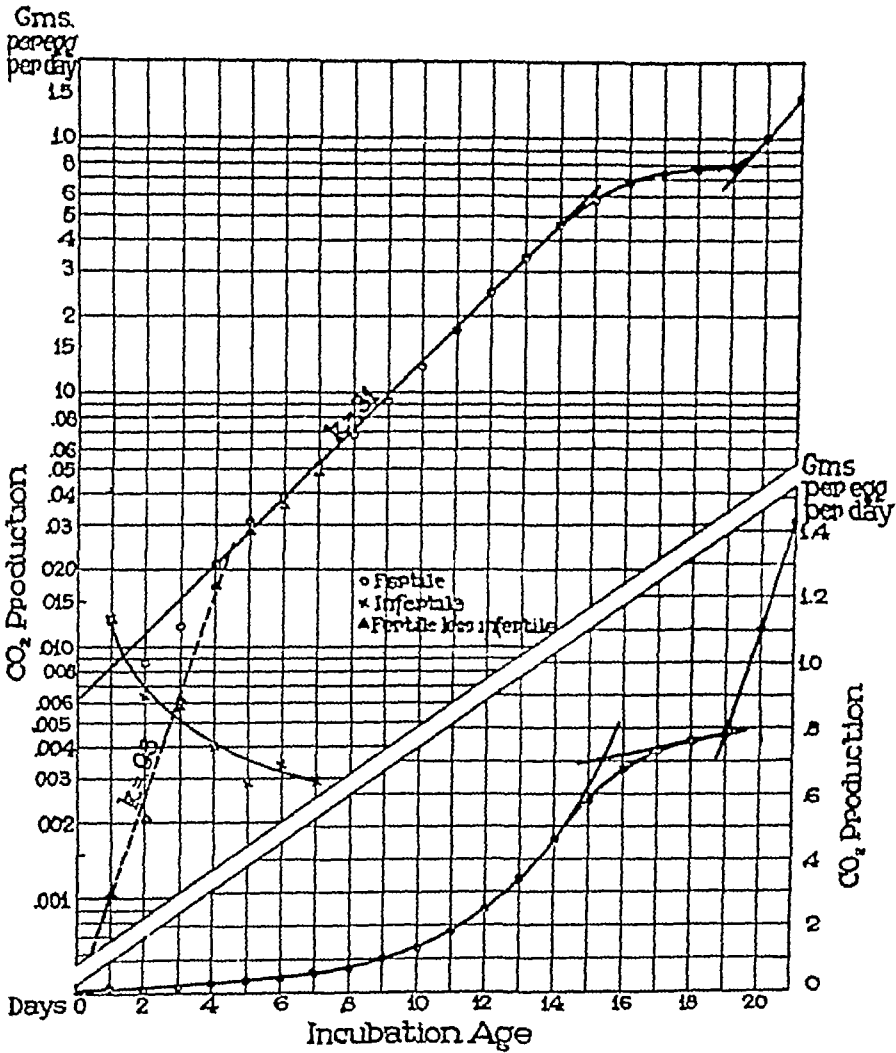


FIG 2 The course of carbon dioxide excretion in the chick embryo with advancing age plotted from data by Atwood and Weakley From 0 to 4 days, the instantaneous percentage rate of growth appears to be 98 per cent per day (the amount of carbon dioxide excretion is doubled once in 7 day, or once in 17 hours), between 4 and 14 days, the rate of increase in carbon dioxide excretion is 31 per cent per day (it is doubled once in 2.2 days) The pause in the curve coincides with the maximum in the mortality curve (of Fig 3), and with the change in the mode of respiration (see text)

the organism, against age, since the integrated form of (2) may be written

$$W = Ae^{kt}$$

Therefore

$$\ln W = \ln A + kt$$

If the data points of the logarithms of weights plotted against age are distributed about a straight line, then the percentage rate of

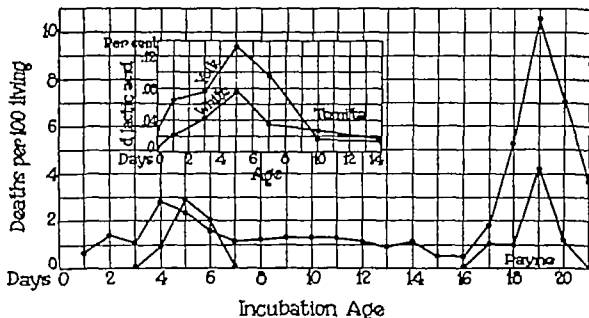


FIG 3 Age changes in the percentage mortality with increasing age in the chick embryo. Open circles represent mortality data of embryos incubated in an incubator; solid circles represent incubation under a hen. The first peak in the mortality curve corresponds with the peak in the concentration of lactic acid as found by Tomita. The second peak in the mortality curve coincides approximately with the pause in the growth curves (Figs 2 and 5).

growth, represented by $100k$ is constant, and k is the growth constant we are seeking. Instead of plotting logarithms of weights, we may plot the data on paper on which the axis of ordinates is divided logarithmically (i.e. on arithlog paper).

IV

The Results of Plotting Growth Data on Arithlog Paper

Fig 2 represents the course of increase in carbon dioxide excretion with age in the chick embryo, as plotted on arithlog paper. The rate

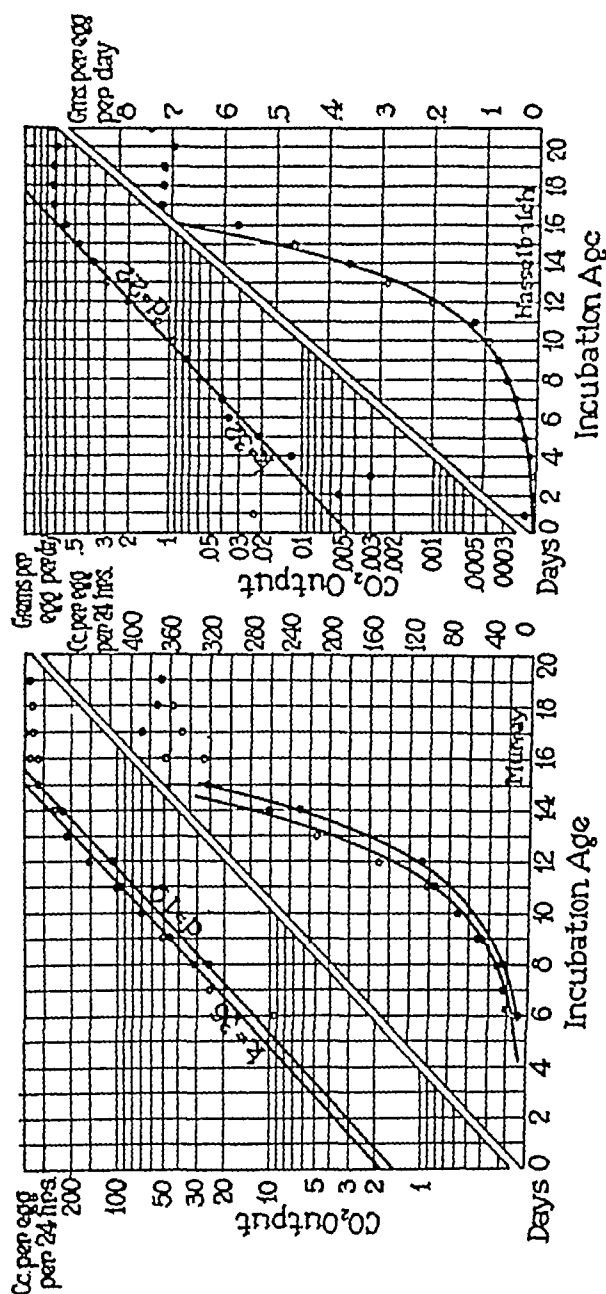


FIG 4 The course of carbon dioxide excretion in the chick embryo, plotted from data by Hasselbalch and Murray d refers to the time in days in which the magnitude of the carbon dioxide excretion is doubled

of growth, as measured by the increase in carbon dioxide production, is constant between the 1st and 4th day of incubation. The increase is of the order of 100 per cent per day, and not 1000 per cent as postulated by Minot. From the 4th to the 15th day, the increase is likewise constant, it is 31 per cent per day.

The pause in the curve between 17 and 19 days, is, no doubt, associated with the change in the mode of respiration (from the aquatic

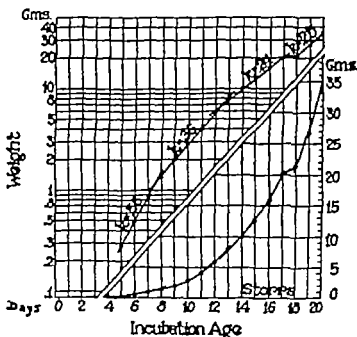


FIG 5 Growth in wet weight of the chick embryos plotted from data by Lamson and Edmond (Storrs Agricultural Experiment Station). The curve appears to consist of four segments each of which represents growth at a constant percentage rate. The pause in the curve between 17 and 18 days coincides approximately with the second peak in the mortality curve (Fig 3). Growth at an instantaneous rate of 56 per cent per day indicates that the body weight is doubled once in 12 days, at 36 per cent per day the body weight is doubled once in 19 days, at 24 per cent per day the body weight is doubled once in 29 days.

to the terrestrial mode) which takes place at this time. Fig 3, representing the course of mortality, likewise presents a disturbance at this time. The peak of mortality at 5 days also represents a critical period as indicated by the presence of a peak in the lactic acid curve.

These results are quite unexpected and no doubt, new. Students of animal growth have accepted the notion of Minot that the per

centage rate of growth declines in a continuous manner with age. The breaks in the curves substantiate, in a way, the "human metamorphosis" conception recently advanced by Davenport.

Fig. 4 represents the course of carbon dioxide production plotted from data by other investigators. The differences between the curves in Figs. 2 and 4 are due to differences in experimental procedure.

Figs. 5, 6, and 7 represent the curves of growth in weight of the chick embryo. There are differences between the weight and the carbon dioxide curves, which leave room for discussion. There are also differences between the weight curves as plotted from data by different investigators. These differences are probably due to differences in the experimental technique employed, especially differences in incubation temperatures. That differences in temperature bring about changes in the growth rates, especially in the earlier stages of incubation, is illustrated by Fig. 8.

During postnatal life, the fowl grows at 5 per cent per day up to 3 weeks, and at 3 per cent from 3 to 12 weeks. The major inflection in the curve takes place at the age of about 12 weeks.

It may be noted in this connection that the rat, guinea pig, cow, sheep, and probably other domestic animals, grow at approximately the same percentage rate during the juvenile period (the stage preceding the major inflection), namely, 2 to 3 per cent per day. Man, however, grows during this period at quite a different (much lower) percentage rate.

The results obtained with the domestic fowl were practically duplicated, as far as the available data permitted, with the rat, guinea pig, cow, sheep, and domestic pig. The data of the rat are of special interest on account of the break in the curve at birth as shown in Fig. 9. It is probable that there is a break in the curve at birth in all classes of animals.

The curve of man differs in several important respects from the curve of animals. The curve of man requires a more extensive discussion than can be given at this time. For this reason a separate paper will be devoted to the growth curve of man.

The curve of plants is similar to that of animals. However, the inadequacy of the data and the relatively large experimental errors

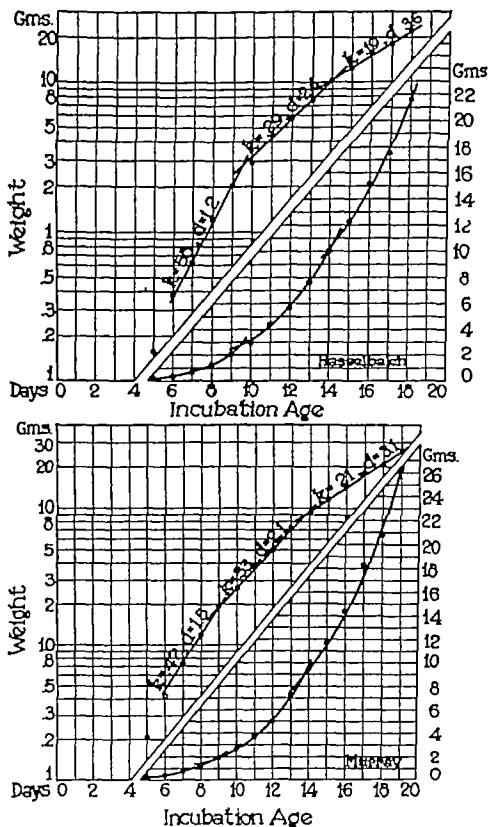


FIG. 6 Growth in wet weight of the chick embryo plotted from data by Hasselbalch and by Murray. The value of $100k$ represents the instantaneous percent age rate of growth per day. The values of d represent the time in days required for the body to double its weight.

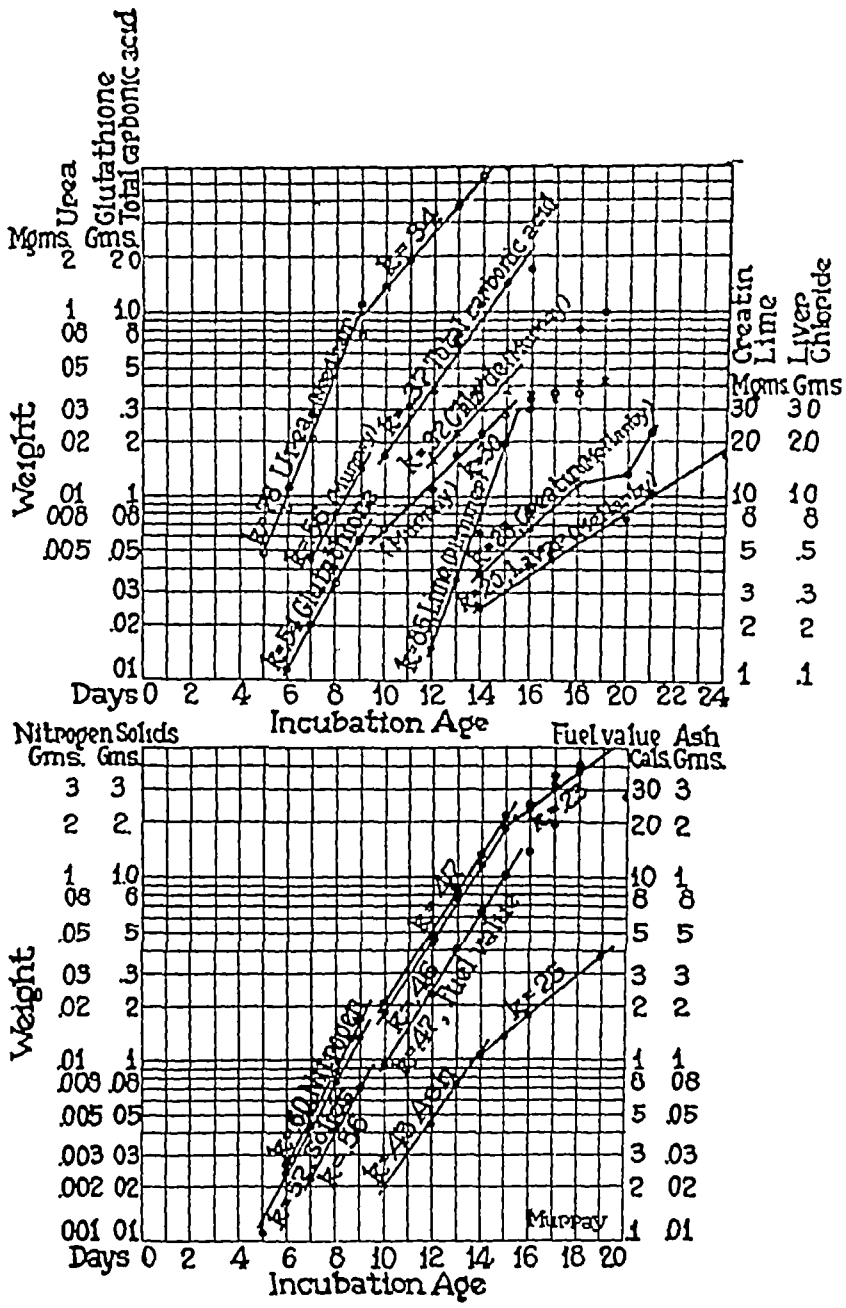


FIG 7 The course of increase in chemical constituents in the chick embryo with advancing age The sources of data are indicated on the chart (compare with Figs 5 and 6)

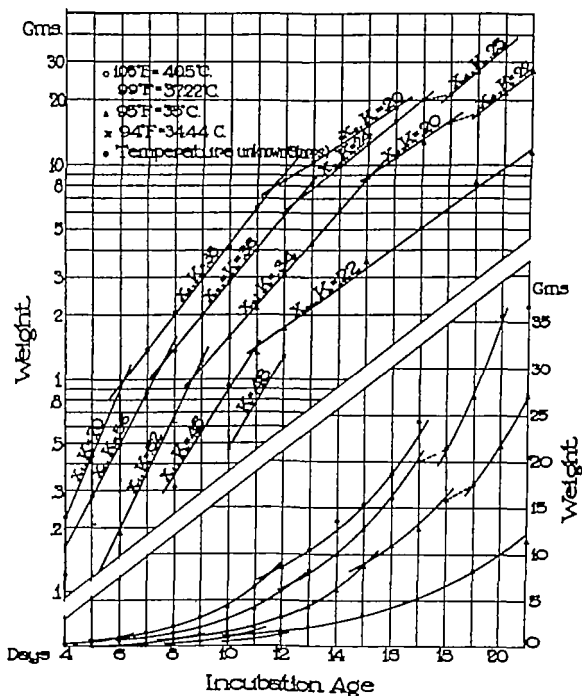


FIG. 8 The effect of temperature on the course of growth of the chick embryo (E. W. Henderson and S. Brody)

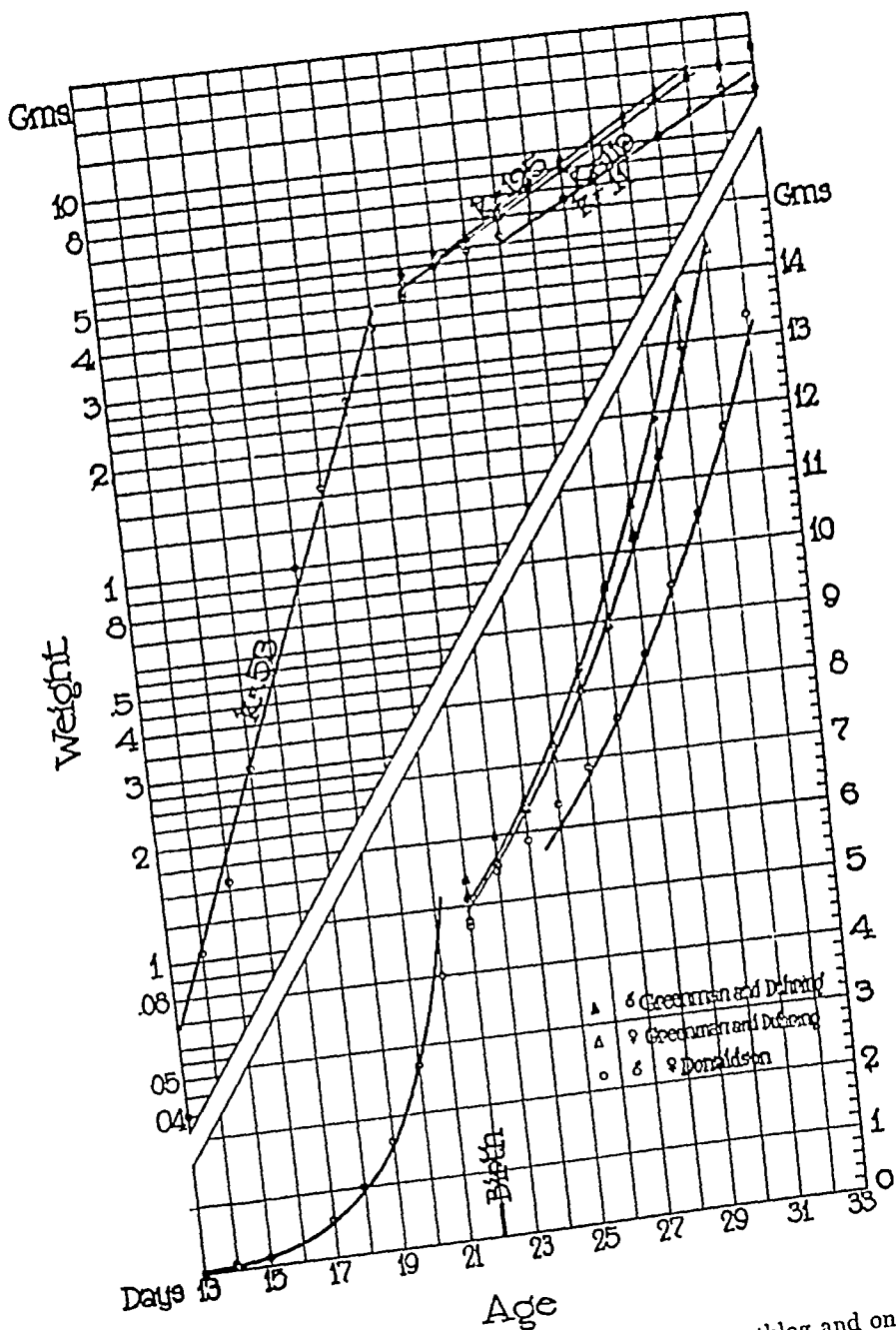


FIG 9 The course of growth of the white rat plotted on arithlog and on coordinate paper. On the arithlog paper the data points are distributed around a straight line indicating that the percentage rate of growth is constant. There is an abrupt break in the curve at the time of birth and the percentage rate is seen to drop from 53 to about 12 per cent. Data preceding birth by Stotsenburg

involved in the investigation of plant materials do not permit formulating conclusions as definite as with animals

Fig 10 shows the course of growth of the wheat kernel. The development of the seed corresponds, in time, to the prenatal growth in

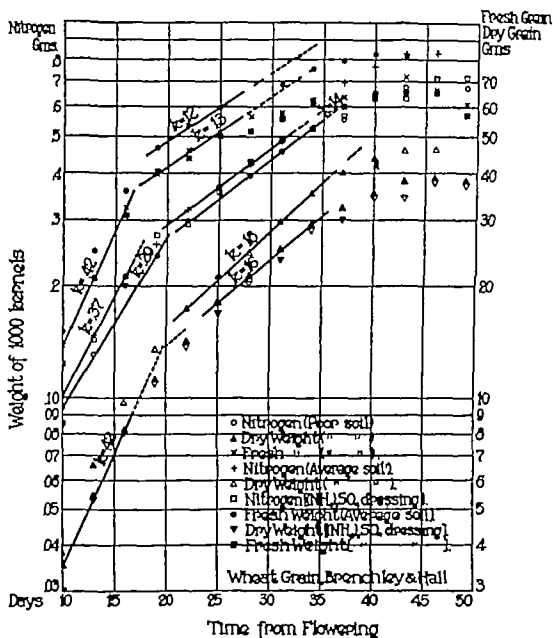


FIG 10 Growth of the wheat grain. k and d have the usual meaning

animals. However, it so happens that in the wheat kernel the embryo is a small fraction of the whole seed (about one-thirteenth), and so the data represent more than embryonic growth

Fig 11 represents the period of independent growth of the maize plant The segment preceding flowering corresponds to the juvenile period in animals, and as in animals, the percentage rate of growth is constant The inflection occurs at the time of flowering, which corresponds to puberty in animals The major inflection in the curve

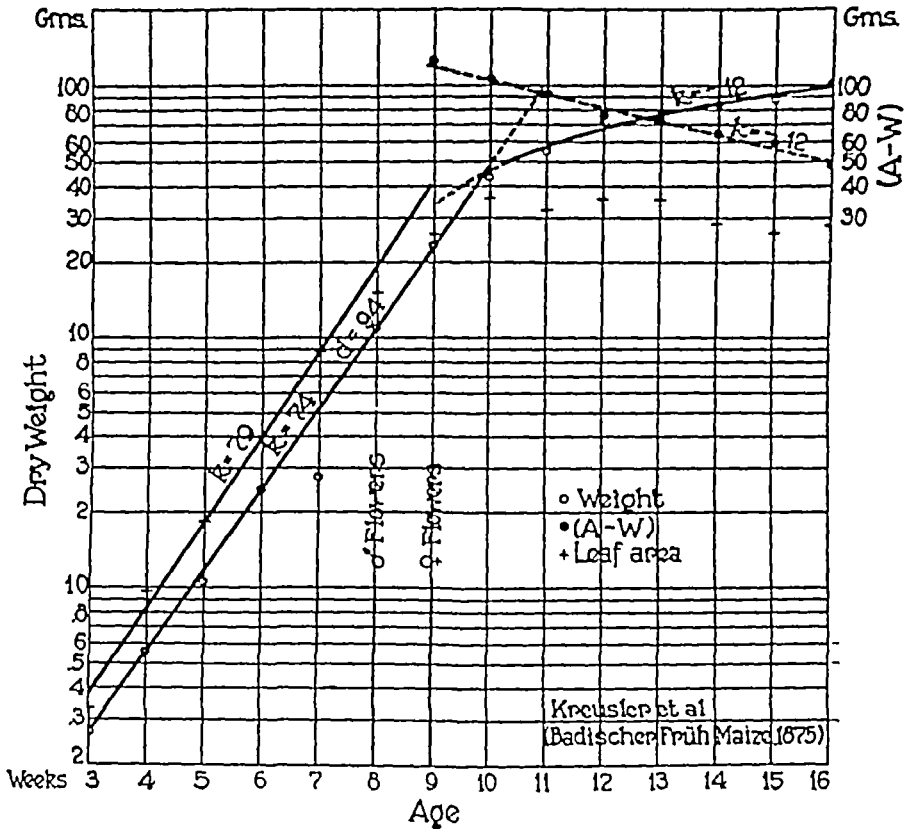


FIG 11 The course of growth of the maize plant (note that the week is the unit of time in this case)

invariably occurs at the time of flowering in higher plants, and at puberty in higher animals

Figs 12 and 13 represent, respectively, the growth of bacterial and human populations The rate of growth is constant during the period preceding the major inflection

As to the bearing of this work on the problem of growth cycles, the

situation, as it appears to the writer, is as follows. All curves pass through an inflection which joins the strictly self-accelerating phase with the strictly self-inhibiting phase of growth. For this period,

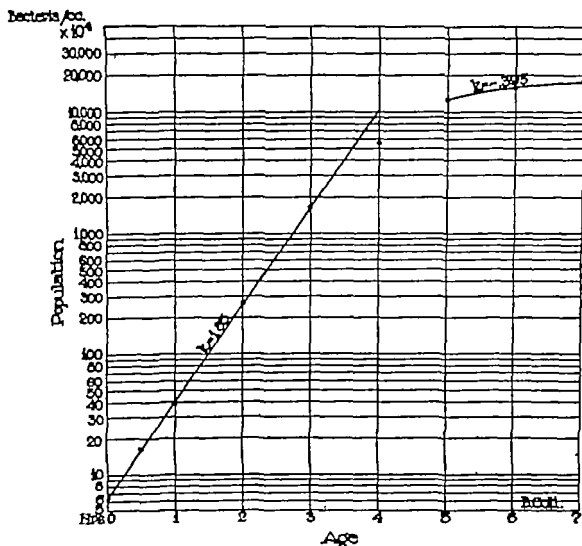


FIG. 12. The course of growth of *B. coli* at 37°C in a given volume of broth (data from Experiment 7 of McKendrick and Pal). The value of k , 1.85, indicates the population of bacteria increases at 1.85 per cent per hour. That is, the population doubles itself every $69/1.85 = .37$ hours or 22.2 minutes. Following the 5th hour, the percentage rate of growth is constant with respect to the growth yet to be made.

which is relatively short, equation (6), the "autocatalytic" equation of Robertson, or the "logistic" equation of Pearl, can be fitted satisfactorily, especially, if a constant, or constants, is employed to compensate for the asymmetric nature of the curve. This equation can

not, however, be satisfactorily fitted to the infantile (except in man), or to the juvenile cycle

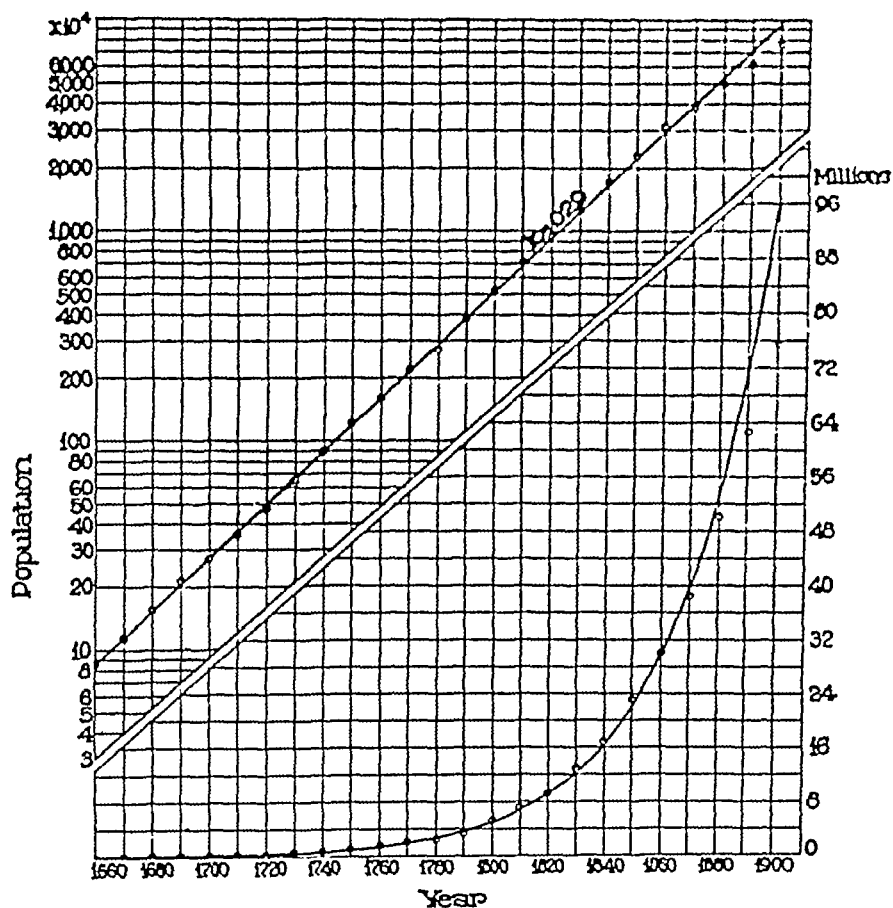


FIG 13 The course of growth of the human population in the American Colonies and in the United States The percentage rate is constant from 1660 to 1870 From 1870 on, the percentage rate declines in a manner indicated by the preceding figure on the growth of bacteria $k = 0.29$, the population increased 2.9 per cent per year, or 29 per cent per decade, or it doubled itself once in $693 / 0.29 = 24$ years (Plotted from data by Rossiter, W S, A century of population growth in the United States Bureau of the Census, United States Department of Commerce and Labor, Washington, 1909)

What we appear to have during the phase of growth preceding the inflection is a series of segments during each of which growth takes

place at a constant percentage rate. These segments are separated by breaks, analogous to the breaks in the curves of cold blooded animals when undergoing metamorphosis. The present need is for growth data taken at shorter intervals in order to ascertain definitely the presence of breaks, and for an investigation of the threshold mechanisms bringing about these breaks, if there are such

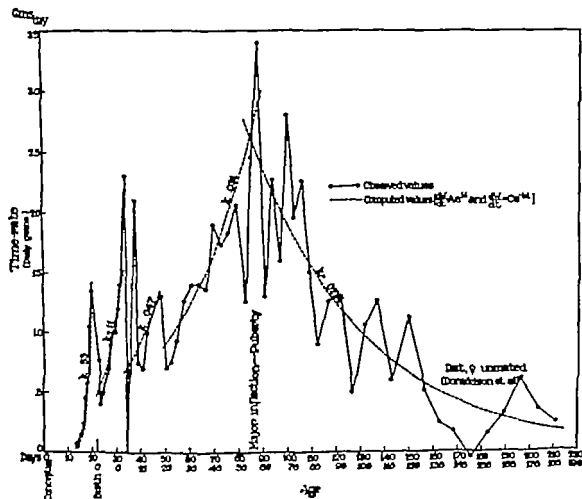


FIG. 14 The daily gains in weight of the rat plotted against age. The curve appears to have three cycles.

When the increments (time rates) are plotted against age, as shown in Fig. 14, there appear to be several cycles preceding the major inflection, as a matter of fact, the drops in the curve are not portions of cycles but breaks between successive stages of constant growth rates, as shown in Fig. 15

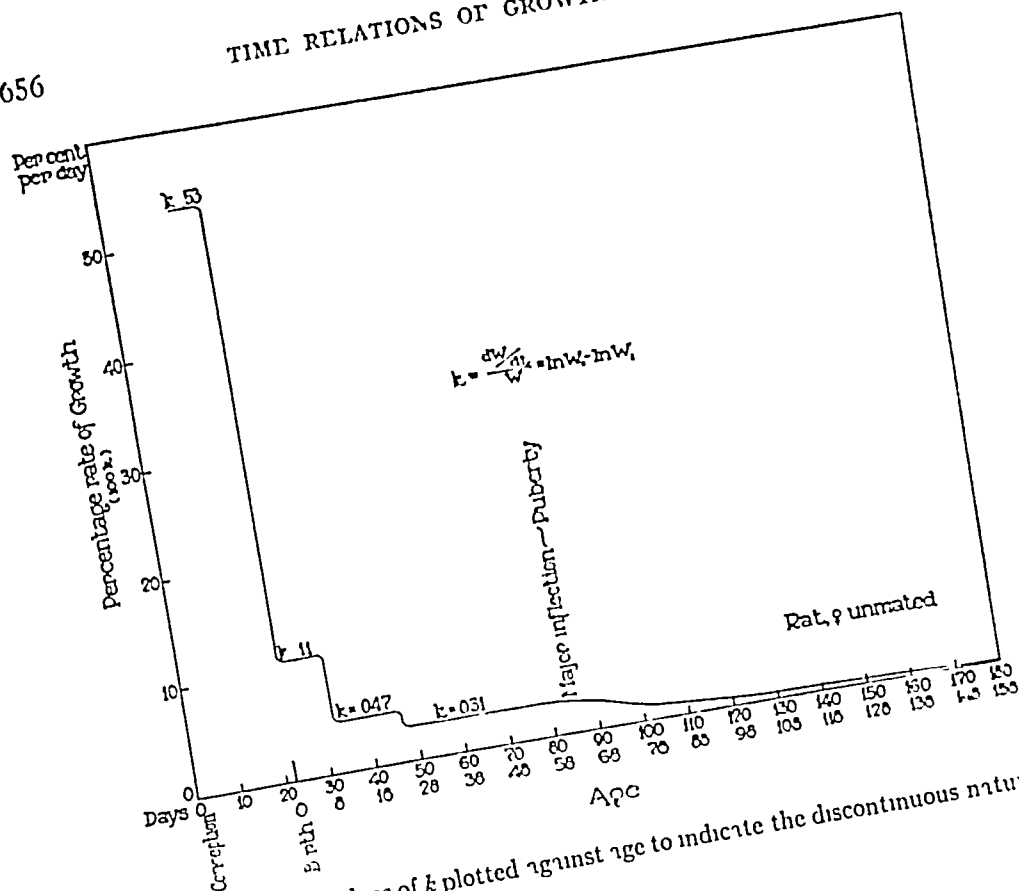


FIG 15 The values of k plotted against age to indicate the discontinuous nature of the growth process

V

CONCLUSIONS AND SUMMARY

Growth curves consist, in all cases, of two major segments. The first major segment is, in the case of higher animals and plants, made up in turn of several (probably five) shorter segments during each of which growth takes place at a constant percentage rate. The transitions between the successive stages are abrupt, the abruptness being of the order of metamorphosis in cold blooded animals. It has been made clear in the first paper of this series that the time rate of growth following the major inflection declines at a constant percentage rate. The junction between the two major segments occurs at puberty in animals and flowering in plants.

The two major segments are not symmetrical about the major inflection. The slope of the segment following the inflection is always less than the slope of the segment preceding the inflection. The major inflection does not occur in the center of the growth curve.

The instantaneous rate of growth at the beginning of growth is of the order of 100–200 per cent per day (i.e. the body weight is doubled in from 7 to 17 hours). It may be mentioned that 2 months after conception the rate of growth in man is only 8 per cent per day. This is contrary to all the published statements. Thus Minot concluded that growth begins at 1000 per cent per day, Jackson concluded that in man, growth during the 1st month takes place at 57.5 million per cent per month, during the 2nd month 990 per cent per month, during the 3rd month 390 per cent per month (8 per cent per day is only 240 per cent per month). The reason for the discrepancy between the values derived, by the method adopted by the writer, and the values given in the literature is explained by Fig. 1.

This paper is a brief summary of Research Bulletins, 97, 98, and 99, of the University of Missouri Agricultural Experiment Station, at present in press. The reader must be referred to these bulletins for detailed discussions relating to questions that may not have been made clear in this paper.

Addendum—Since this manuscript was submitted for publication the writer had the privilege of discussing its subject matter with Drs E. B. Wilson, C. R. Stockard, and H. H. Donaldson, all of whom expressed approval of the two principal ideas. Dr Wilson called attention to a paper by G. H. Knibbs on the Laws of population growth which appeared (on January 8) in the *Journal of the American Statistical Association* 1926, xxi, 381, substantiating in principle one of the two principal ideas of this paper, namely that in the early history of a population the percentage rate of growth is constant. Dr Stockard called attention to the fact that the peak in the mortality curve of the chick (Fig. 3) at 5 days is a counterpart of the peak in the prenatal mortality curve in man at 3 months. This is the junction between the embryonic period (formation of organs) and fetal period (enlargement of body and organs). The nature of the growth process in the two stages is quite different, and it is not, therefore, surprising to find a high mortality (and break in the growth curve) at this time.

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THE KINETICS OF EXOSMOSIS OF WATER FROM LIVING CELLS

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(Accepted for publication February 17 1927)

In previous communications data were presented on the rate with which water enters living cells (the unfertilized egg of *Arbacia punctulata*) under the driving force of osmotic pressure (1, 2) The present paper is concerned with the reverse process—exosmosis of water

The material and technic of the experiments were the same as formerly employed

The Kinetics of Exosmosis

The first point was to determine whether exosmosis follows the same diffusion equation as does endosmosis, namely $\frac{dx}{dt} = k(a - x)$, where a is the total volume of water that will cross the membrane before equilibrium is established, x the amount that has already crossed at time t , and k is the velocity constant For this purpose, eggs were placed in a dish containing 60 per cent sea water (sea water 60 parts, and distilled water, 40 parts) In this hypotonic solution, eggs were allowed to swell until osmotic equilibrium was attained A number of eggs were then transferred to a second dish containing full strength sea water (100 per cent sea water) Three cells were measured with an ocular screw micrometer at minute intervals, until they had again reached osmotic equilibrium Duplicate observations were usually made by the two observers The mean volumes of 6 or more cells were plotted against times, and a curve obtained, as is shown in Fig 1 In the same graph, $\log \frac{a}{a - x}$ is plotted against times This plot is found to give a straight line, the slope of which is k , the velocity constant